Integral action of hypoxia-inducible HIF prolyl-4-hydroxylases in mammalian oxygen sensing

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HABILITATIONSSCHRIFT

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Summary

The elucidation of the molecular mechanisms by which cells respond to insufficient oxygen supply (herein after referred to as "hypoxia") is central to our understanding of many medical processes in health and disease. During embryonic development, adaptation to high-altitudes but also in wound healing and inflammation, the cellular oxygen sensing pathways allow tissues to appropriately adapt their metabolic requirements to an altered oxygen environment. Exhausted capacities of these coping strategies ultimately accumulate in irreversible tissue demise as observed in ischemic diseases such as infarction and stroke. The very same signalling structures, however, are frequently activated in cancerous lesions, supporting an anaerobic hypermetabolic phenotype of the corrupted cells.

Hypoxia-inducible transcription factors (HIFs) have been identified as master regulators of the hypoxic response, directly activating the expression of a diverse set of effector genes involved in anticipatory metabolic changes, adaptive survival, or programmed death of the affected tissue. HIFs are heterodimeric transcription factors composed of oxygen-labile α-subunits and constitutively expressed HIF β-subunits, originally identified as aryl hydrocarbon receptor nuclear translocator (ARNT). Stability of HIF-α is regulated by a proteolytic step requiring post-translational hydroxylation at two distinct proline residues situated in the so-called oxygen-dependent degradation (ODD) domain. The actual O_2-sensing molecules in this process are the recently identified HIF prolyl-4-hydroxylases, alternatively termed prolyl-4-hydroxylase domain (PHD), HIF prolyl hydroxylase (HPH) or egg laying defective nine homolog (EGLN). In an archaic, yet fascinatingly effective reaction, PHDs catalyze the transfer of an oxygen atom derived from molecular O_2 to proline 402 or proline 564 of human HIF-1α, thereby providing a recognition interface allowing the von Hippel-Lindau (pVHL) tumor suppressor to bind and initiate poly-ubiquitination and proteasomal degradation. Facilitating a similar reaction, factor inhibiting HIF (FIH) hydroxylates a C-terminal asparaginyl residue within HIF-α subunits, thereby regulating the transcriptional activity of HIFs.

My personal interest in the emerging field of oxygen sensing focused on the molecular understanding of integrational sites of the complex HIF-pathway, where a variety of certain upstream effector-pathways are translated into a single action. This work summarizes four publications built on each other where I contributed as (co-)first or last author, broadening our understanding of the fundamental role of PHD oxygen sensor proteins as integral regulators of oxygen-sensitive pathways within cells.

A single promoter-embedded HIF-binding site confers hypoxic inducibility to human PHD2

When HIF prolyl-4-hydroxylases were identified as the molecular oxygen sensors in 2001, two out of the three members encompassing family have been shown to be oxygen regulated. Working together with Prof. Metzen (Essen, Germany), we characterized a functional hypoxia response element (HRE) in the promoter of the human *PHD2* gene, providing experimental evidence that PHD2 can be classified as a direct HIF target gene. Approaching a cloning strategy utilizing a bacterial artificial chromosome image clone, we were the first to succeed in cloning of the upstream regulatory region of the human *PHD2* gene. Subsequent studies of this region in luciferase reporter gene assays revealed that a CpG-rich promoter element in close proximity to the translational start site of the gene was sufficient for basal and hypoxia-inducible PHD2 transcription. A putative consensus 5'-ACGTG-'3 HIF-1 binding site conserved amongst humans and mice was identified by bioinformatic sequence analysis. Functional recruitment of HIF-1 to this region was further demonstrated by gel shift experiments and site-directed mutagenesis of the respective site in promoter reporter contructs.

PHD and HIF proteins trigger a feedback loop facilitating adaptive oxygen sensing

Under hypoxic conditions the co-substrate O_2 becomes limiting for PHD enzymes and prolyl-4-hydroxylation ceases, dramatically increasing the protein half-life of HIF-α. Initial reports emphasized a dominating role for PHD2 in setting the normoxic levels of HIF-α and more recently embryonic lethality of PHD2, but not PHD3 and PHD1 knock-out mice confirmed this prominent function of PHD2. In consideration of the striking width of tissue pO_2 values found in healthy organisms even when the inspiratory pO_2 is considered to be "normoxic" (oxygen partial pressures may range from ~100 mm Hg in the lung alveolar cells to <1 mm Hg in avascular cartilage tissue), it appeared enigmatic from a Physiologist's view, how a rather simple post-translational enzymatic modification could comply with such different requirements and function as unique molecular oxygen sensing mechanism.

Puzzled by this problem, my more recent work could demonstrate that hypoxically induced HIF prolyl-4-hydroxylases are well capable of functionally regulating HIF-α subunits even under limited oxygen conditions. Given the opportunity of latest generations' oxygen-controlled incubators to handle and even split cell cultures without perturbation of the external oxygen concentration, we were able to grow cells for extended periods at 1% oxygen. Using such a model system, we showed in a broad range of cell lines that HIF-1 activation indeed is a transient event and cells are capable to adapt to a given oxygen threshold by increasing the hydroxylation capacity of the oxygen sensors PHD2 and PHD3, thus establishing a new base-line, "steady state" activation of the HIF-PHD sensory circuit. Such a self-regulatory gene-effector circuit offers an appealing model, how cellular oxygen sensing is generically functional throughout the whole organism in multicellular life. Obviously, PHD oxygen sensors need to operate at different pO_2 setpoint values - in different tissues as well as in variable environments e.g. when under water or at high altitude. Since PHDs are non-equilibrium enzymes and to date no antagonizing dehydroxylation event has been identified, cellular abundance of the enzymes must be considered as a rate limiting parameter of the HIF-hydroxylation reaction. In keeping with one pre-requisite for a rapidly tunable system, we could show that PHDs are not abundant within cells but a rather fine balance between HIF-α production and PHD-dependent degradation is kept: If either one is upregulated, it overcomes the function of the other. Thus, as well as oxygen availability limits the activity of PHD sensors, an increase in PHD synthesis leads to HIF-α degradation. Likewise, upregulated HIF-α synthesis will temporarily exceed the preset hydroxylation capacity of a cell, ultimately leading to its stabilization even under oxic conditions. If tissue oxygenation is constant, the definition of a setpoint for oxygen sensing by adjusting the HIF/PHD ratio will keep the system tuned and allow a secondary response to a further, more severe hypoxic insult.

A reconstituted *in vitro* system as a tool to study PHD dependent HIF-α hydroxylation

PHDs belong to the superfamily of 2-oxoglutarate (2-OG) and Fe(II)-dependent di-oxygenases catalysing the oxidative de–carboxylation of 2-OG to succinate by releasing gaseous CO_2. In support with a classical model of product inhibition, succinate (and other Krebs cycle intermediates) have been reported to interfere with PHD hydroxylation activity, providing a possible link between mitochondrial respiration and cellular oxygen sensing pathways. Likewise, a variety of bi-valent transition metal ions, known since the early days of hypoxia research to phenocopy cellular responses to oxygen deprivation, have been suggested to exert their "hypoxia mimicking" effects *via* interference with HIF-hydroxylating PHDs. In order to systematically study the effects of putative PHD–inhibitory and -activating compounds, a reconstituted *in vitro* hydroxy-lation assay was adapted to our needs. In brief, this assay utilizes recombinant PHDs as enzyme source to hydroxylate a synthetic, biotinylated peptide derived from human

HIF-1α coupled to strepavidin-coated 96-well plates. The fraction of hydroxylated peptide is subsequently quantified by enzyme-linked immunosorbent detection of bound recombinant pVHL in complex with Elongins B and C. As full length PHD enzymes expressed and purified from bacterial cultures happened to maintain poor enzymatic activity, we established an insect-cell based recombinant expression system for all isoforms of PHDs. We could show, that all three PHDs purified close to homogeneity were similarly dependent on addition of exogenous ferrous iron and reduced vitamin C (ascorbate) in the micromolar range to allow efficient *in vitro* hydroxylation of HIF-α peptides. Moreover, *N*-acetyl-L-cystein was unable to replace ascorbate in this *in vitro* reaction and some antioxidants even inhibited HIF-α hydroxylation (as the polyphenolic compounds found in green tea). Yet, a more specific function of ascorbate in supporting PHD activity than simply providing antioxidative moieties has to be considered. Underlining its important function *in vitro*, our data further revealed that metal-catalyzed oxidation of vitamin C (e.g. by cupric copper) completely abolished PHD function, providing a plausible mechanism for the HIF-stabilizing effects of at least some transition metals.

Cellular oxygen sensing is not affected by nutritional vitamin C deficiency

It was broadly accepted that HIF-hydroxylases are vitamin C dependent enzymes, a view that is supported by the requirement of vitamin C for sufficient *in vitro* hydroxylation activity of PHDs as observed by several groups, including ours. Given the close relationship of PHDs to collagen prolyl-4-hydroxylases (C-P4H), where a clear link between systemic vitamin C deficiency, reduced C-P4H activity and the collagen disassembly-disease scurvy (lat. *scorbutus*) is well known, we speculated that PHDs might likewise be affected by vitamin C abundance *in vivo*. This assumption was further supported by the relatively high *Km* values for both, PHDs and C-P4H, ranging between 140 and 300 μM ascorbate, respectively. Of note, human cells in culture have much lower intracellular ascorbate concentrations (1-50 μM) than these *Km* values, and plasma levels of vitamin C in humans are in a similar range.

To our surprise, a fully functional oxygen sensing pathway was found in cells adapted to grow in the complete absence of vitamin C. Supporting these data, we found no significant differences in the systemic response to inspiratory hypoxia between control and scorbutic animals of a genetically modified mouse strain resembling the pathophysiology of scurvy. In parallel studies using our hydroxylation assay platform we had identified glutathione (GSH) as a strikingly potent activator of PHD enzymes with the abilities to fully substitute ascorbate in the hydroxylation reaction *in vitro*. Thus, this highly abundant intracellular antioxidant might have compensated the lack of ascorbate as a co-factor for PHDs in our *in vivo* model systems. Indeed we could show, that GSH and ascorbate were similarly functional to reduce HIF-1α accumulation in response to the hypoxia-mimetic cobalt chloride in human hepatoma cultures (though both of the antioxidants failed to impair hypoxic HIF-1α induction). As hypoxia-mimetics unphysiologically activate the HIF pathway at oxic conditions, we further tested the influence of oxidative stress to the PHD enzymes itself. A profound reduction of protein carbonylation was noted when GSH was present in the hydroxylation reaction, suggesting that oxidative damage to the enzyme during hydroxylation reaction might well affect the activity of PHDs. Wether such a model also applies to HIF-α hydroxylation by PHDs *in situ*, where the intracellular environment must be considered as strongly reducing, requires further work. In summary, our work could clearly show that vitamin C is not an essential co-factor for HIF-α hydroxylation by PHD enzymes *in vivo*, challenging the current view of PHDs as vitamin C requiring enzymes.

Regulation of the prolyl hydroxylase domain protein 2 (*phd2*/*egln-1*) gene: identification of a functional hypoxia-responsive element

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The HIFs (hypoxia-inducible factors) are a family of heterodimeric transcription factors essential for the adaptation of cells to reduced oxygen supply. Three human PHDs (prolyl hydroxylase domain proteins, PHD1–PHD3) initiate oxygen-dependent degradation of HIF-α-subunits in normoxia. RNA interference directed against PHD2, but not PHD1 or PHD3, is sufficient to stabilize HIF-1α in normoxia. Therefore PHD2 is regarded as the main cellular oxygen sensor. PHD2 itself is up-regulated by hypoxia and may thus limit hypoxic signalling. By sequence analysis, we predicted a promoter approx. 3.5 kb 5′ of the translation start codon and a second promoter located in a CpG island immediately upstream of the coding sequence. A consensus HIF-1-binding site that is conserved in the murine *phd2* gene was detected in the CpG island. By electrophoretic mobility-shift assay, we demonstrated binding of HIF-1 to the putative HIF-1-binding site. In luciferase reporter vectors, the isolated upstream promoter was inactive in all cell lines tested unless 200 bp were deleted at the 3′-end. The downstream promoter was active and induced by hypoxia. In reporter vectors containing both promoter sequences, luciferase activity was equal to vectors containing only the downstream promoter. In cells transfected with a vector containing both promoters, a single luciferase transcript was detectable. This transcript had the same length as transcripts from a vector containing the downstream promoter only. We conclude that the *phd2* gene is transcribed exclusively from the downstream promoter that contains a functional hypoxia-responsive, *cis*-regulatory element. Our results establish that PHD2 is a direct HIF target gene.

Key words: egg-laying deficiency protein nine-like protein (EGLN), hydroxylation, hypoxia, hypoxia-inducible factor (HIF), oxygen, prolyl hydroxylase domain (PHD).

INTRODUCTION

HIF (hypoxia-inducible factor) is a transcription factor central to a large number of adaptive processes in a situation of reduced oxygen supply. Hypoxia is a feature of cardiovascular diseases associated with ischaemia and also plays an important role in tumour biology. Therefore HIF is currently regarded as an attractive target in the development of new therapeutic strategies (see [1–3] for reviews).

HIF is composed of an oxygen-sensitive α- and a constitutive β-subunit that is also known as arylhydrocarbon receptor nuclear translocator. Three related α-subunit paralogues have been characterized. For example HIF boosts red blood cell production by the induction of erythropoietin, and enhances transcription of angiogenic factors, e.g. vascular endothelial growth factor. Furthermore, HIF induces glucose transporters as well as virtually all glycolytic enzymes and thus favours anaerobic cell metabolism. More than 50 genes have been found to be HIF responsive (see [4] for a review). Inducibility by HIF is linked to the presence of a core HIF binding consensus sequence (ACGTG) in regulatory regions of HIF target genes. The most prominent example of a HIF binding HRE (hypoxia-responsive element) is the enhancer located 3′ to the *epo* gene that enabled the identification of HIF-1 [5,6].

The α-subunit of the active transcription factor HIF-1 is normally undetectable in the presence of oxygen whereas it becomes stable in hypoxia. The degradation process is triggered in normoxia by enzymatic hydroxylation of two conserved proline residues (Pro-402 and Pro-564 in human HIF-1α, [7–9]). Only hydroxylated HIF-α binds the pVHL (von Hippel–Lindau protein) that is part of an E3 ubiquitin ligase. After ubiquitination, HIF-α molecules are rapidly degraded by the proteasome [10,11]. A second control mechanism is the hydroxylation of Asn-803 in the C-terminal transactivation domain by the enzyme FIH-1 (factor inhibiting HIF-1) [12–14] that prevents the recruitment of transcriptional co-activator proteins.

The four human HIF-α hydroxylases that have been characterized so far (PHD1–PHD3 and FIH-1, where PHD stands for a prolyl hydroxylase domain protein) belong to a family of 2-oxoglutarate-dependent, non-haem iron-binding dioxygenases [15–17]. PHD1, PHD2 and PHD3 have also been termed HPH3, HPH2 and HPH1 [16], and EGLN2, EGLN1 and EGLN3 [18]. No substrates other than HIF-α have been reported to date. The PHDs are widely expressed in tissues [19,20]. PHD homologues, as well as HIF homologues, have been identified in all multicellular organisms investigated so far, termed EGL-9 (egg-laying deficiency protein 9) in *Caenorhabditis elegans* [15], CG1114 in *Drosophila melanogaster* [21] and SM-20 in rat [22]. In mouse, three PHDs with similarity to the human enzymes have been found by database analysis [18].

Notably, PHD2 inhibition by RNAi (RNA interference), but not inhibition of PHD1 or PHD3, is sufficient to up-regulate HIF-1α in normoxia, indicating that the three enzymes are not simply redundant [23] and that PHD2 may be the main cellular oxygen sensor. PHD2 and PHD3 have been reported to be hypoxia-inducible [15,23,24]. Dysregulation of HIF-α in pVHL-deficient

Abbreviations used: EGLN, egg-laying deficiency protein nine-like protein; EMSA, electrophoretic mobility-shift assay; EST, expressed sequence tag; FIH, factor inhibiting HIF-1; HIF, hypoxia-inducible factor; HBS, HIF-1-binding site; HRE, hypoxia-responsive element; MEF, mouse embryonic fibroblasts; PHD, prolyl hydroxylase domain protein; pVHL, von Hippel–Lindau protein; RNAi, RNA interference; UTR, untranslated region; wt, wild-type.

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renal clear cell carcinoma cells is associated with the loss of hypoxic PHD2 and PHD3 induction [25], and the inhibition of HIF-α by RNAi also leads to a loss of induction of PHD2 and PHD3 in hypoxia [26]. These results suggest that HIF itself may mediate the induction and that HIF and PHDs form a feedback loop that limits hypoxic signalling and accelerates HIF degradation after reoxygenation. However, HIF-responsive elements in control of the *phd2* gene have not been identified yet.

In the present study, we demonstrate that a promoter element located in a CpG island approx. 0.5 kb upstream of the translation start site of the *phd2* gene is dominant in all cell lines tested. This promoter contains a HBS (HIF-1-binding site) that is the *cis*-acting regulatory element causing induction of the human *phd2* gene by hypoxia. Thus our results prove that PHD2 is a direct HIF target gene.

MATERIALS AND METHODS

Computational sequence analysis

Promoter region prediction and analysis of transcription factor binding sites in relevant parts of human and murine genes for PHD2 were performed with the GenomatixSuite software (Genomatix Software GmbH, Munich, Germany). Sequence alignments were performed with the tools of ClustalX program [27]. CpG islands within the genes of human and murine *phd2* were identified by means of the CpG-island-extraction algorithm as described previously [28]. The CpG island searcher is available at http://www.uscnorris.com/cpgislands/cpg.cgi. EST (expressed sequence tag) searching was performed using the Gene2EST BLAST server available at http://zurg.embl-heidelberg.de/

Cell culture

Human hepatoma cells HepG2, human ovarial carcinoma cells OVCAR3 and osteosarcoma cells U2OS were cultured in Dulbecco's modified Eagle's medium (Gibco, Karlsruhe, Germany) supplemented with 10 % (v/v) foetal calf serum (Gibco), 2 mM glutamine (Gibco), 50 units/ml penicillin and 50 μg/ml streptomycin (Sigma, Deisenhofen, Germany). Normal $MEF^{+/+}$ (mouse embryonic fibroblasts) and the same cells bearing an inactivation of the *hif-1α* gene ($MEF^{-/-}$) were engineered by R. S. Johnson (Division of Biological Sciences, University of California at San Diego, La Jolla, CA, U.S.A.) and provided by R. H. Wenger (Institute of Physiology, University of Zürich, Zurich, Switzerland). MEFs were cultured in the same media as the human cells. Hypoxic incubations were performed either in an oxygen-regulated cell culture incubator (Heraeus, Hanau, Germany) or in an $InvivO_2$ 400 hypoxia workstation (Ruskinn Technologies, Leeds, U.K.).

Nuclear extract preparation and EMSA (electrophoretic mobility-shift assay)

Nuclear extracts were prepared from HepG2 cells incubated in a 3 % oxygen atmosphere for 4 h as described earlier [29]. Oligonucleotides for gel-shift assays were synthesized by MWG-Biotech (Ebersberg, Germany). Sequences were derived from the human *phd2* gene, containing the putative HBS (PHD2-HBS, nt 2747, GenBank® accession no. AF229245) or a mutated HBS (PHD2-HBSmut). Sequences for wt (wild-type) and mutant oligonucleotides were as depicted in Figure 1(A). As positive control, an oligonucleotide containing the HIF-1 responsive element (containing two HBSs) from the human transferrin gene (TfHBS) was used as described in [30]. The 5′-end labelling, annealing and binding reactions were performed as described previously [29]. Samples were resolved by electrophoresis on native 5 % polyacrylamide gels at room temperature (20 °C). Gels were dried and analysed by phosphoimaging (BAS 1000; Fuji, Düsseldorf, Germany). Specificity was tested by supershift experiments. For this purpose, 1 μl of undiluted monoclonal anti-HIF-1α antibody (BD Biosciences, Heidelberg, Germany) was added 60 min before the gel was run.

Quantitative PCR

Mouse embryonic fibroblasts were incubated in an atmosphere of 1 % oxygen for 4 h. Total RNA was isolated following a standard method [31]. RNA (1 μg) was reverse transcribed with oligo-(dT) and Transcriptor Reverse Transcriptase (Roche, Penzberg, Germany). The coding sequence of mouse PHD2 was assembled from ESTs published previously [18]. Primer sequences were: (forward) 5′-GACCGGCGTAACCCTCATG-3′ and (reverse) 5′-TTGCTGACTGAATTGGGCTTG-3′. Mouse PHD2 mRNA expression was quantified using the qPCR Mastermix for SYBR Green I (Eurogentech, Seraing, Belgium) and the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, U.S.A.). Murine ribosomal *L28* was used as a housekeeping gene. PCRs were set up in a final volume of 25 μl and contained 2 μl cDNA, 1× reaction buffer with SYBR Green I and 20 pmol of each forward and reverse primer. PCR conditions were set to 10 min at 95 °C followed by 40 PCR cycles (15 s at 95 °C and 1 min at 60 °C). Amplification of one specific PCR product was confirmed by melting-point analysis. Data were analysed using the delta-delta Ct method (where Ct stands for cycle threshold).

Plasmid construction

Fragments of the *phd2* gene used in this study were amplified by PCR using DNA isolated from bacterial artificial chromosome clone RP11-295G20 (GenBank® accession no. AL445524; Wellcome Trust Sanger Institute, Hinxton, U.K.) provided by RZPD Deutsches Ressourcenzentrum für Genomforschung GmbH (Berlin, Germany). Luciferase reporter gene constructs were obtained by cloning the region of interest into the pGL3-family of vectors (Promega/Boehringer Ingelheim, Heidelberg, Germany). Mutations of the HBS plasmids were produced by site-directed mutagenesis utilizing the oligonucleotide primers PHD2-HBSmut (sense) and PHD2-HBSmut (antisense) that had also been used for EMSA. Since the HBS is located in a CpG-rich region, mutagenesis PCR was performed with Thermal Ace® polymerase (Invitrogen, Karlsruhe, Germany) for amplification of GC-rich templates according to the manufacturer's instructions. The numbering of all plasmids is according to the mRNA registration by Dupuy et al. (GenBank® accession no. AF229245) [33]. All plasmids containing PCR-inserts or mutations respectively were sequence-verified (Seqlab, Goettingen, Germany).

In detail, the following plasmids were constructed: for the subset of 5′-promoter reporter vectors, a region of −1535 to +1 flanked XhoI and HindIII was cloned into pGL3-basic. This sequence contains an internal HindIII site. Thus incomplete digestion with HindIII resulted in pGL3b(−1535/1)P2P and pGl3b-(−1535/−191)P2P. A SacI–XhoI PCR-fragment spanning nt −985 to +137 was ligated into pGL3-basic and was named pGL3b(−985/137)P2P. For pGl3b(1454/3172)P2P-wt, the designated PCR product flanked XhoI and HindIII was amplified and ligated into pGL3-basic. The corresponding plasmid pGl3b-(1454/3172)P2P-mt containing the mutation of the HBS was generated as described above.

Reporter constructs possessing the two putative regulatory regions in the endogenous context [pGL3b(−1535/3172)P2P-wt and pGL3b(−1535/3172)P2P-mt respectively] were derived by a two-step cloning strategy. A XhoI–BamHI flanked PCR-product

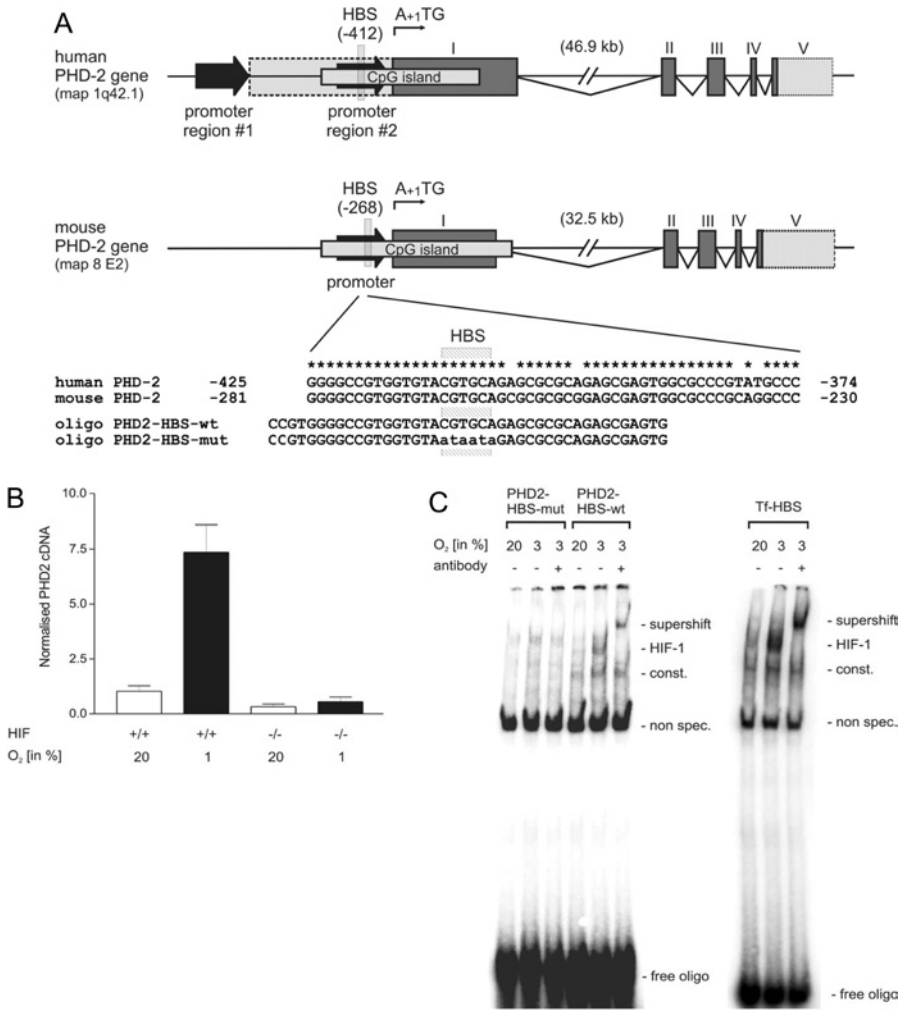

Figure 1 HIF-dependence of PHD2 expression

(**A**) The *phd2/egln-1* gene in human and murine genome. Although in mouse the analysis of nucleotide sequences recognizes a single promoter located in a CpG island closely upstream of the translational start site, analysis of the human gene leads to prediction of one promoter 3.5 kb upstream of the translational start site and an alternative promoter in a CpG island approx. 0.5 kb 5′ of the translational start site. The HBS is conserved between species. (**B**) PHD2 expression in mouse embryonic fibroblasts containing wt ($HIF^{+/+}$) or inactivated ($HIF^{-/-}$) *hif-1α* gene. The cells were incubated in normoxia (20 % O_2) or hypoxia (1 % O_2). Total mRNA was subjected to reverse transcription and quantitative PCR. Relative amount of PHD2 cDNA was calculated by the delta-delta Ct method. Individual PHD2-Ct values were normalized to corresponding L28 Ct-values. Normalized PHD2 values were set in relation to the mean of the $HIF^{+/+}$ normoxia group. (**C**) EMSAs with nuclear extracts from HepG2 cells. Cells were incubated at 20 or 3 % oxygen for 4 h. Oligonucleotides used were: PHD2-HBSmut (mutated HBS, negative control), PHD2-HBS-wt (putative HBS) and Tf-HBS (positive control). Specificity of the HIF-1 signal was demonstrated by supershift analysis with an anti-HIF-1α antibody. HIF-1, specific signal; const., constitutive DNA-binding; non spec., non-specific DNA-binding. All samples were run on the same gel. The Tf-HBS part of the image was electronically brightened.

corresponding to (−1535/1454) was ligated into pGL3-basic utilizing an internal BamHI site at 1454 of the *phd2* gene. Subsequently, a cassette containing (1454/3172) and the *luc* gene was released with BamHI from pGL3b(1454/3172)P2P-mt and cloned into the intermediate to gain the full-length vector. The corresponding wt was obtained by replacing an endogenous SfiI fragment including the wt HRE derived from SfiI-digested pGl3b(1454/3172)P2P-wt.

Reporter gene plasmids carrying the HRE-regulatory region in *trans*-position, i.e. downstream of an SV40 promoted luciferase gene, were derived by a recombinant cloning strategy. An internal BamHI site in position 1454 was used to release a BamHI–XhoI fragment of pGl3b(1069/3172)P2P-wt and of pGl3b(1069/3172)-P2P-mt and ligated into pGL3-prom opened with BamHI–SalI. The resulting plasmids were termed pGl3-prom-3′E(1454/3172)-P2P-wt and pGl3-prom-3′E(1454/3172)P2P-mt respectively. Purity and concentration of all plasmids transfected into cells was estimated by agarose gel electrophoresis.

Transfection and luciferase assays

Transient transfections were performed on 100 mm dishes using Fugene 6 (Roche Applied Science, Mannheim, Germany) transfection reagent by following the manufacturer's instructions. To normalize for transfection efficiency, 3 μg of each reporter construct was co-transfected with 1.5 μg of a cytomegalovirus-promoter-driven β-GAL expression plasmid (a gift from P. J. Ratcliffe, The Henry Wellcome Building of Genomic Medicine, University of Oxford, Oxford, U.K.). Transfected cultures were grown overnight, trypsinized the following day and split into two equal parts and placed on two 6-well plates, i.e. from a single transfected plate 12 separate cell culture wells were produced which allowed the incubation of cells from the same transfection either in normoxia or in hypoxia. Cells were allowed to become adherent and subsequently incubated for 16 h either under normoxic or hypoxic (1 % O_2) conditions. Cell lysates were prepared with passive lysis buffer (Promega) and aliquots were assayed

for luciferase expression with a Microlumat LB96P luminometer (EG-G Berthold, Bad Wildbad, Germany). Additionally, β-GAL expression for each sample was measured. Data are expressed in relative light units (luciferase counts/β-GAL expression). Bars indicate means $\pm$ S.D. for six separate cell culture wells. All experiments were performed repeatedly so that all results were confirmed in at least one completely independent experiment.

Transient transfections for subsequent luciferase Northern blotting were performed following a similar transfection procedure. Cells growing in 100 mm dishes were transfected with 3 μg of the indicated reporter constructs. Plates were grown overnight and divided equally on two 150 mm culture dishes. Corresponding cultures were exposed to either normoxia or 1 % oxygen for 16 additional hours, cells were then lysed and total RNA was isolated as described above.

Northern blotting

Human osteosarcoma U2OS cells and human ovarial carcinoma cells OVCAR3 were incubated in a normoxic (20 % O_2) or hypoxic (1 % O_2) atmosphere for 4 h. Total RNA was isolated as described above. mRNA was purified using 0.5 mg of total RNA (Oligotex mRNA Midi; Qiagen, Hilden, Germany) following the manufacturer's instructions. mRNA (4 or 6 μg) of each sample was subjected to electrophoresis in denaturing 1 % agarose gels containing 0.7 M formaldehyde. RNA transfer, prehybridizations and hybridizations were performed exactly as described previously [29]. A hybridization probe was generated by restriction digest of pcDNA3-PHD2 with NotI and EcoNI and thus contained nt 3543–3800 (GenBank® accession no. AF229245). Ribosomal protein L28 mRNA was used to confirm equal loading and transfer as described previously [32]. The L28 hybridization probe was amplified from HepG2 cDNA by PCR. For detection of luciferase mRNA, 20 μg of total RNA was resolved on denaturing agarose gels. The hybridization probe for luciferase mRNA was excised from pGL3-basic using EcoNI and thus encompassed nt 646–1046 (GenBank® accession no. U47295). In this case equal loading was verified by ethidium bromide staining of 28 and 18 S rRNA. All probes were labelled using a commercial DNA labelling kit (MBI Fermentas, St. Leon Rot, Germany). Filters were analysed by phosphoimaging.

RESULTS

The prime objective of the present study was to identify the HRE(s) conferring hypoxic induction of the *phd2*/*egln-1* gene. Interestingly, two GenBank® entries have been deposited (GenBank® accession nos. AF229245 and AJ310543) that do not differ regarding the coding sequence of PHD2. However, one of these sequences contains an unusually large 5′-UTR (5′-untranslated region) [33], which has been confirmed experimentally by anchored PCR using an embryonic cDNA as template. The other has been assembled from ESTs and has a short 5′-UTR but an extended 3′-UTR [18].

To define regulatory sequences, we analysed genomic DNA from approx. 8 kb upstream relative to the translation start site to a region approx. 5 kb downstream of the human PHD2 coding sequence. Two putative promoter regions were predicted, one of which contains a cellular TATA box and is located 3.5 kb upstream of the translation start site, whereas the second promoter region is contained in a CpG island 0.5 kb 5′ of the translation start codon. Transcription factor binding analysis identified one HIF-binding motive (ACGTG) located in the downstream promoter 412 bp upstream of the start codon (Figure 1A).

Importantly, the potential HBS within the CpG island is conserved in mouse *phd2* sequence. In line with this computational analysis, we show by comparison of mouse embryonic fibroblasts carrying a null mutation for the HIF-1α gene with wt cells [34] that the hypoxic induction of mouse *phd2* expression is exclusively dependent on the presence of HIF-1α (Figure 1B).

To demonstrate the functional importance of the putative HBS, it was a prerequisite to prove binding of HIF-1α to the sequence under investigation. In EMSAs, oligonucleotides derived from the human *phd2* sequence containing the wt HBS bound a protein complex from hypoxic HepG2 cells which was supershifted on the addition of a HIF-1α antibody (Figure 1C). Binding of HIF-1 was abolished by mutation of the HBS.

We went on to dissect the function of the two putative promoters, to analyse their interplay, and their oxygen sensitivity by means of luciferase reporter gene assays. The promoter regions were cloned separately or in combination, 5′ to a promoterless luciferase gene. The expression of the luciferase gene was analysed after normoxic and hypoxic incubation. In all cell lines tested, the upstream promoter was inactive. In contrast, the downstream promoter was moderately active in normoxia but strikingly induced by hypoxia (Figure 2A). In all cell lines, hypoxic induction was dependent on the presence of the HBS located in the CpG island. We also used vectors that contained both putative promoters and demonstrated that the activity of this construct was essentially indistinguishable from constructs containing only the downstream promoter region (Figure 2B). When we deleted the terminal 200 bp of the upstream promoter it became active but remained unresponsive to hypoxia (Figure 2C). This result may indicate that the promoter region between -200 and $+1$ contains a repressor element that blocks promoter activity in all cell lines that we have analysed. To further delineate the properties of the downstream promoter region, we produced a vector that contained the luciferase gene driven by a heterologous SV40 promoter followed by the regulatory sequence in a 3′ position. In this experiment, the downstream sequence conferred hypoxic inducibility on the SV40 promoter, i.e. the region under investigation can, at least in this artificial reporter gene context, have enhancer function (Figure 2D).

We have noted that independent groups have found two bands by Northern blotting for PHD2 in human [33] and in murine [20] tissues. These transcripts had a length of approx. 4 and 2 kb respectively. Another report shows that three bands are detectable [23], which is consistent with our own PHD2 Northern blot results (Figure 3A). Interestingly, our experiments showed that all of these bands were induced by hypoxia.

To determine whether transcripts are produced from the upstream promoter when it is not in isolation, we transfected cells with luciferase vectors that contained both promoter regions or the isolated downstream promoter and performed luciferase Northern blotting. We detected a single transcript that had the same length in all samples, indicating that all transcripts originated from the downstream promoter (Figure 3B).

The activity of a single hypoxia-responsive promoter was in harmony with the observation that all transcripts were hypoxia-inducible, but it did not help to interpret the presence of three detectable PHD2 transcripts by Northern blotting. Revisiting the GenBank® entries AF229245 and AJ310543, we found that three poly(A)$^+$ (polyadenylated) signals have been reported which are located 60, 500 and 2630 bp downstream of the translation stop signal. The use of the downstream promoter together with these alternative poly(A)$^+$ signals is predicted to result in three transcripts, which are approx. 1.9, 2.3 and 4.4 kb in length, which is well in line with our Northern blotting results. To test this hypothesis, we used U2OS mRNA samples for reverse transcription

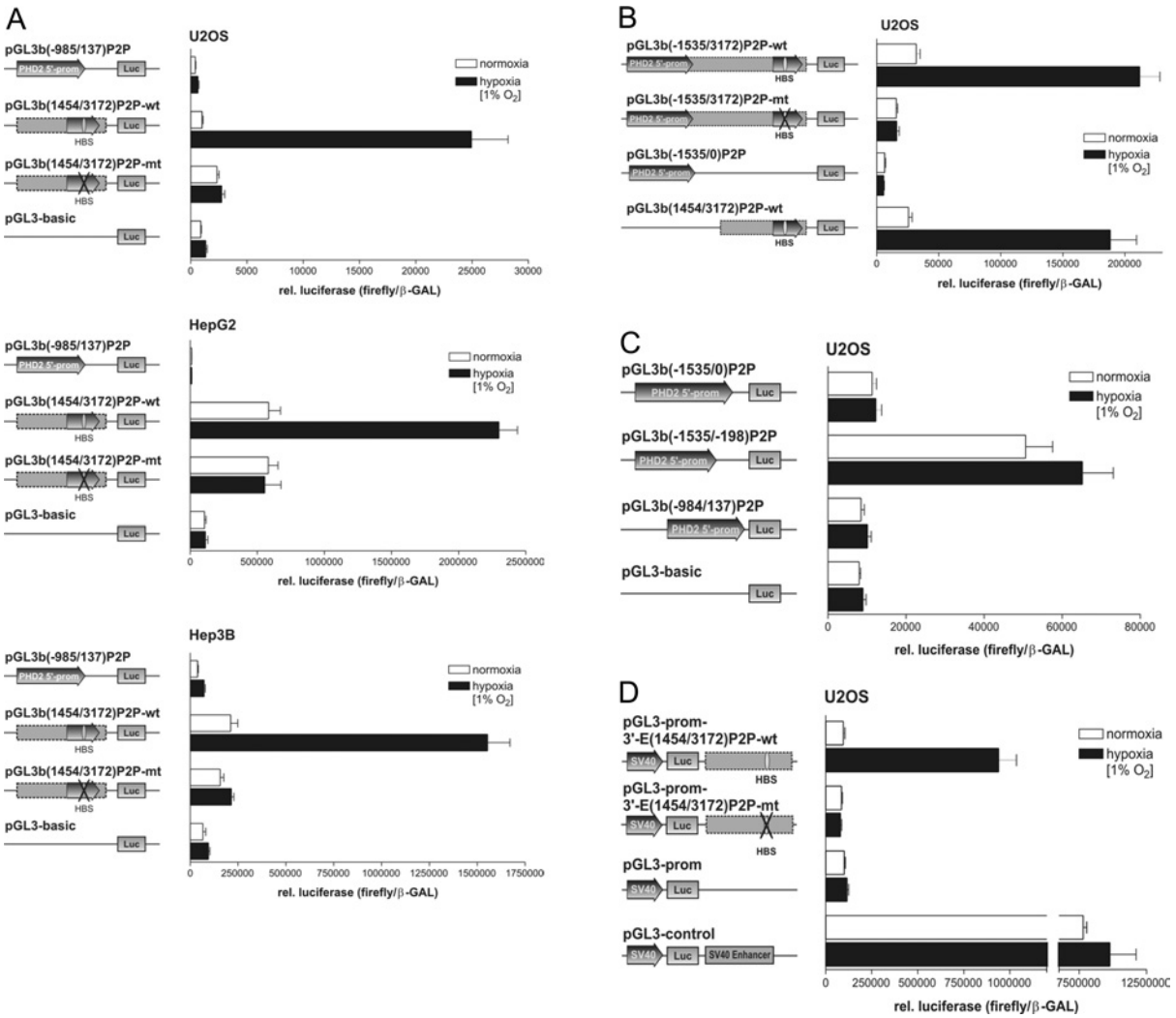

Figure 2 Luciferase reporter gene assays

The regulatory DNA regions of the human *phd2* gene were cloned into luciferase reporter vectors, which were transiently transfected into cells. 24 h after transfection, cells were incubated overnight under normoxia or hypoxia. β-galactosidase served as a transfection control. Bars represent means $\pm$ S.D. for six separate cell culture wells. (**A**) The function of the promoter located approx. 3.5 kb upstream of the translational start site (PHD2 5′-prom) when compared with the downstream promoter region which contains either the wt HBS (wt) or a mutation of the HBS (mt) in U2OS, HepG2 and Hep3B cells. For example, a pGL3-basic vector that contains position -985 to position $+137$ of the putative regulatory region of PHD2 (numbering according to GenBank® accession no. AF229246) is termed pGL3b(-985/137)P2P. (**B**) Luciferase expression was analysed in U2OS cells transfected with reporter vectors containing both regulatory regions or the isolated downstream promoter element. (**C**) The isolated upstream promoter was compared with a truncated version of the same regulatory region. (**D**) The downstream promoter region in a position downstream of the luciferase gene conferred hypoxic induction on an SV40 promoter, demonstrating enhancer activity of the region under investigation.

with oligo(dT) or specific oligonucleotides binding upstream of the 3′ promoter. Although we could readily amplify PCR products from the extended 3′-UTR, we were not able to generate PCR products from the region between the two promoters (results not shown). These results suggest that indeed only the downstream promoter is active in cells and that the different transcripts arise from the use of distinct poly(A)$^+$ signals.

DISCUSSION

The human HIF-α prolyl-4-hydroxylase PHD2 is of high biomedical relevance since it is the enzyme that down-regulates the oxygen-sensitive transcription factor subunit HIF-α [23] in normoxia. Besides the reports mentioned above further studies have demonstrated the induction of PHD2 and of another HIF-prolyl-4-hydroxylase (PHD3) by hypoxia in various cell lines and by use of different experimental techniques [35–37]. It has also been shown that dysregulation of HIF-1α is associated with the loss of hypoxic induction of PHD2. Inactivation of pVHL, a protein necessary for oxygen-dependent degradation of HIF-1α, leads to high normoxic levels of HIF-1α and PHD2 [25]. RNAi directed against HIF-1α has also been reported to eliminate hypoxic PHD2 induction [26]. These results suggest that PHD2 may be a target gene of HIF-1. The evidence is, however, circumstantial. The classification of PHD2 as a HIF target gene requires the experimental identification of a *cis*-regulatory HRE in the *phd2*/*egln-1* gene, which was the aim of the present study. Importantly, presence of a consensus HIF-binding motif in a regulatory DNA domain is not sufficient to demonstrate hypoxic induction by HIF, as it has been shown that erythroid 5-aminolevulinate synthase is hypoxia-inducible but HIF-independent although a putative HBS is found in the promoter of this gene [38].

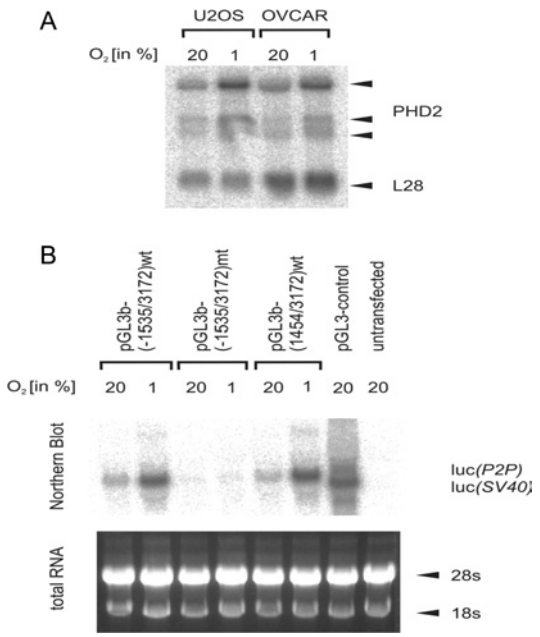

Figure 3 Analysis of PHD2 promoter function by Northern blotting

(**A**) PHD2 Northern blot. Human osteosarcoma (U2OS) and ovarian carcinoma (OVCAR3) cells were subjected to normoxic (20% O_2) or hypoxic (1% O_2) incubation for 4 h. mRNA was isolated as described in the Materials and methods section. mRNAs (4 μg U2OS or 6 μg OVCAR3) were run in each lane. Hybridization probes corresponded to PHD2 nt 3543–3800, GenBank® accession no. AF229245. The PHD2 transcripts are approx. 4.1, 2.3 and 1.8 kb. The constitutively expressed ribosomal protein L28 was used as a loading and transfer control. (**B**) Luciferase Northern blot U2OS cells were transfected as described in the Materials and methods section. The cultures were subjected to normoxic or hypoxic incubation overnight. Total RNA was isolated and 20 μg was resolved in each lane.

The *phd2* gene has been localized as the 12th open reading frame on chromosome 1 (C1ORF12) and is reported to contain five exons [33]. According to the report, exon 1 includes 4047 bp with the translational start site in position 3157, thus the initial 3156 bp are an unusually large 5′-UTR. Importantly, this 5′-UTR has been verified experimentally by PCR walking and anchored PCR using a random embryonic total cDNA library. Consequently, the study implies that an active promoter is situated upstream to the 5′-UTR. However, searching EST databases with the 5168 bp PHD2 mRNA sequence (GenBank® accession no. AF229245) results in retrieval of more than 300 ESTs none of which contains a sequence upstream to the CpG island overlapping the translation start site.

Presence of a CpG island spanning the terminal region of the 5′-UTR and the beginning of the coding sequence is a hallmark of mammalian promoters [39,40]. Consequently, promoter prediction programs recognize this region as a second promoter. Interestingly, this prediction is in line with a second GenBank® PHD2 mRNA sequence assembled from ESTs (GenBank® accession no. AJ310543), which gives a 5′-UTR of approx. 100 bp.

Embedded in this CpG island, we have identified a putative HBS, by sequence analysis, situated −412 bp relative to the translational start site. Interestingly, the HBS as well as the surrounding sequence is conserved in the murine *phd2* gene (Figure 1A), which suggests functional relevance. Indeed we have shown that hypoxic induction of murine *phd2* is dependent on HIF-1.

Our sequence analysis suggested that two promoters are located upstream of the PHD2 coding sequence. The upstream promoter element which is located approx. 3.5 kb 5′ of the transcription start site is inactive in our experimental setting. Interestingly, truncation of this promoter region had an activating effect. It seems possible that a repressor element inhibited transcription initiation by this promoter region. Our results do not preclude the possibility that this promoter is active in a different cellular background or at a different stage of development. The dominant promoter, however, is situated in a CpG island immediately 5′ of the translation start site and contains a functional HRE. The different transcripts detected by Northern blotting in previous reports [20,23,33] and in our experiments are explained by the use of three separate poly(A)$^+$ signals. The possibilities for the production of distinct PHD2 transcripts are summarized in Figure 4.

Two further reports add complexity to the situation: alternative splicing has been described in [41] for exons 3 and 4. We have not reproduced these data, but as these exons span 207 bp only, alternative splicing cannot account for the different transcripts we have noted. Secondly, the existence of a gene termed *scand2* that has probably evolved by retroposition of the *phd2* gene has been demonstrated [33]. *Scand2* is located on chromosome 15q25, and several transcript variants have been reported. The functions of this gene as well as its regulation are unclear so far. However, since large blocks of nucleotide sequence are highly conserved, it seems mandatory to align the *phd2* and *scand2* sequences and to choose hybridization probes and oligonucleotide primers from unique regions to avoid confounding results.

In essence, we have identified the dominant promoter of the *phd2* gene 0.5 kb 5′ of the translational start site. The promoter

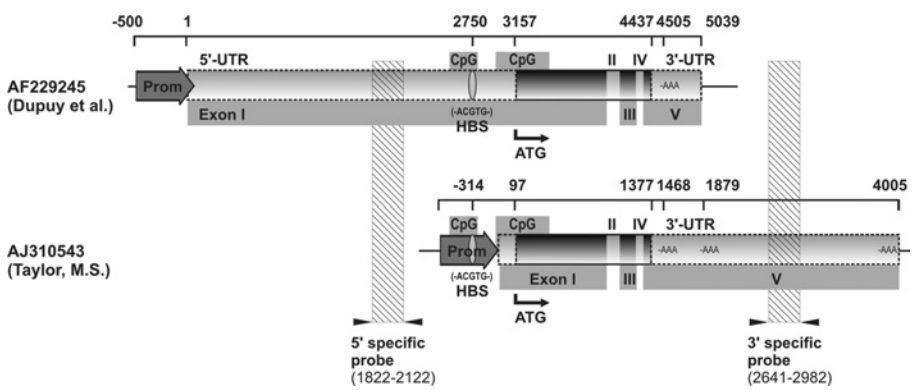

Figure 4 Illustration of the two GenBank® entries potentially leading to distinct PHD2 mRNA species

A transcript containing the extended 5′-UTR and the extended 3′-UTR would encompass 7.5 kb and has not been detected yet. The '5′-specific probe' and '3′-specific probe' designate regions of the *phd2* gene used to detect transcripts containing the extended 5′-UTR and the extended 3′-UTR respectively.

is located in a CpG island, contains a functional HBS, and thus confers hypoxic inducibility. By identification of this *cis*-acting regulatory element, PHD2 is classified as a direct HIF target gene. As PHD2 seems essential in the regulation of HIF-1, our findings may add to the understanding of mammalian oxygen sensing.

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Increased Prolyl 4-Hydroxylase Domain Proteins Compensate for Decreased Oxygen Levels

*EVIDENCE FOR AN AUTOREGULATORY OXYGEN-SENSING SYSTEM**[S]

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Prolyl 4-hydroxylase domain (PHD) proteins are oxygen-dependent enzymes that hydroxylate hypoxia-inducible transcription factor (HIF) α-subunits, leading to their subsequent ubiquitination and degradation. Paradoxically, the expression of two family members (PHD2 and PHD3) is induced in hypoxic cell culture despite the reduced availability of the oxygen co-substrate, and it has been suggested that they become functionally relevant following re-oxygenation to rapidly terminate the HIF response. Here we show that PHDs are also induced in hypoxic mice *in vivo*, albeit in a tissue-specific manner. As demonstrated under chronically hypoxic conditions *in vitro*, PHD2 and PHD3 show a transient maximum but remain upregulated over more than 10 days, suggesting a feedback down-regulation of HIF-1α which then levels off at a novel set point. Indeed, hypoxic induction of PHD2 and PHD3 is paralleled by the attenuation of endogenous HIF-1α. Using an engineered oxygen-sensitive reporter gene in a cellular background lacking endogenous HIF-1α and hence inducible PHD expression, we could show that increased exogenous PHD levels can compensate for a wide range of hypoxic conditions. Similar data were obtained in a reconstituted cell-free system *in vitro*. In summary, these results suggest that due to their high O_2 K_m values, PHDs have optimal oxygen-sensing properties under all physiologically relevant oxygen concentrations; increased PHDs play a functional role even under oxygen-deprived conditions, allowing the HIF system to adapt to a novel oxygen threshold and to respond to another hypoxic insult. Furthermore, such an autoregulatory oxygen-sensing system would explain how a single mechanism works in a wide variety of differently oxygenated tissues.

Biological systems tightly monitor acute changes in environmental conditions, initiate regulatory responses, and use negative feedback loops to limit the extent of these responses. To adapt to chronic changes, many environmental sensors are capable of adjusting their threshold values, allowing to respond again to acute deviations of a now different set point.

Cells sense changes in environmental oxygen availability by a group of enzymes that directly control the cellular response to lowered oxygen by destabilizing hypoxia-inducible factor (HIF)[2] α subunits, the master transcriptional regulators of the hypoxic response. These oxygen-sensing enzymes have alternatively been termed prolyl 4-hydroxylase domain (PHD), HIF prolyl hydroxylase (HPH), or egg laying defective nine homolog (EGLN). The following three family members are known up to date: PHD1/HPH3/EGLN2, PHD2/HPH2/EGLN1, and PHD3/HPH1/EGLN3 (1–3). PHDs hydroxylate HIF-1α and HIF-2α at two distinct proline residues within the HIFα oxygen-dependent degradation (ODD) domain. Under normoxic conditions, prolyl 4-hydroxylation allows binding of the von Hippel-Lindau tumor suppressor protein (pVHL), leading to polyubiquitination and proteasomal destruction (4). Under hypoxic conditions, prolyl 4-hydroxylation is reduced, and HIF-1α and HIF-2α become stabilized, heterodimerize with the constitutively expressed HIF-1β subunit aryl hydrocarbon receptor nuclear translocator (ARNT), and regulate the expression of a large number of effector genes involved in adaptation to low oxygen (5). In addition, factor inhibiting HIF hydroxylates a C-terminal asparagine residue, thereby regulating the transcriptional activity of HIFs (6–8).

Upon re-oxygenation, the PHD oxygen-sensing system must be rapidly reversed. Interestingly, PHD2 and PHD3, but not PHD1, have been reported to be hypoxically induced at both the mRNA and protein levels (2). Accordingly, elevated PHD2 and PHD3 levels have been demonstrated in a broad panel of established cancer cell lines (9). Functional hypoxia-response elements are located in the promoter region of the human *PHD2* gene as well as in the first intron of the human *PHD3* gene, suggesting that *PHD2* and *PHD3* are HIF target genes themselves (10, 11). Because the essential co-factor oxygen is basi-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1 and supplemental Fig. 1.

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[2] The abbreviations used are: HIF, hypoxia-inducible factor; ARNT, aryl hydrocarbon receptor nuclear translocator; CA, carbonic anhydrase; F_i, inspiratory oxygen fraction; GLUT, glucose transporter; mAb, mouse monoclonal antibody; MEF, mouse embryonic fibroblast; ODD, oxygen-dependent degradation; PHD, prolyl 4-hydroxylase domain; pVHL, von Hippel-Lindau tumor suppressor protein; VBC, pVHL/elongin B/elongin C; siRNA, short interfering RNA; RT, reverse transcription; GST, glutathione *S*-transferase; HPH, HIF prolyl hydroxylase; EGLN, egg laying defective nine homolog; DMOG, dimethyloxalylglycine.

cally lacking under hypoxic conditions, the HIF-dependent hypoxic increase in PHD abundance has been suggested to play a role in accelerating the termination of the HIF response following re-oxygenation (2, 9, 12, 13). Indeed, biochemical *in vitro* studies revealed K_m values of purified PHDs for oxygen close to the oxygen partial pressure (pO_2) in air, suggesting that the kinetics of specific HIFα hydroxylation under hypoxic conditions are rather slow (14). However, tissues *in situ* have to deal with a great variability of generally very low pO_2 values, even when the inspiratory pO_2 is considered to be "normoxic." We therefore raised the question whether HIF-dependent regulation of PHD levels might lead to the adaptation of the PHD-HIF oxygen-sensing system to a given tissue pO_2, rather than simply accelerating HIFα destruction following re-oxygenation. Such a self-regulatory loop might define a tissue-specific threshold for HIFα activation as a function of local pO_2.

EXPERIMENTAL PROCEDURES

Cell Culture—All cells were maintained in Dulbecco's modified Eagle's medium-high glucose, containing 4.5 mg/ml glucose (Sigma) to maintain cellular energy metabolism during prolonged hypoxic culturing. Culture media were supplemented with 10% heat-inactivated fetal calf serum, 50 IU/ml penicillin, and 50 μg/ml streptomycin (Invitrogen). Mouse embryonic fibroblast (MEF)-*Hif1a*$^{+/+}$ and MEF-*Hif1a*$^{-/-}$ and mouse hepatoma Hepa1 and Hepa1c4 were cultured as described before (15, 16). For long term hypoxia, MEFs were grown under 2% O_2 for up to 256 h in a gas-controlled glove box to allow for handling of the cells under constant pO_2 (Invivo$_2$ 400, Ruskinn Technologies, Leeds, UK). Cells were grown on 145-mm culture dishes and split every 48 h. Reagents used for splitting and permanent culturing were pre-equilibrated to the pO_2 in the glove box before use. For oxygen titration, a single batch of transfected MEF-*Hif1a*$^{-/-}$ cells was distributed into different hypoxic incubators (Binder, Tuttlingen, Germany) and simultaneously cultured for the indicated time periods.

Protein Extractions and Immunoblot Analyses—Cells were washed twice and scraped into ice-cold phosphate-buffered saline. Soluble cellular protein was extracted with a high salt extraction buffer containing 0.1% Nonidet P-40 essentially as described before (17). Protein concentrations were determined by the Bradford method, and 50–80 μg of protein were subjected to immunoblot analysis. Antibodies were obtained from the following sources: anti-human HIF-1α mouse monoclonal antibody (mAb), BD Transduction Laboratories; anti-human PHDs rabbit polyclonal antibodies, Novus Biologicals (Littleton, CO); anti-V5 tag mAb, Invitrogen; anti-β-actin mAb, Sigma; horseradish peroxidase-coupled secondary antibodies, Pierce. Anti-human PHD3 mAb was kindly provided by P. J. Ratcliffe (Oxford, UK). Bound antibodies were detected with ECL substrate (Pierce), and chemiluminescence was quantified with a CCD camera-based light imaging system (FluorChem8900, AlphaInnotech, Witec, Littau, Switzerland) using QuantityOne software (Bio-Rad).

mRNA Quantification—Exposure of mice to inspiratory 0.1% carbon monoxide or 7.5% oxygen and serum erythropoietin quantifications by radioimmunoassay have been published previously (18–20). Total RNA from tissue and cells was purified as described before (17). RNA concentrations were determined spectrophotometrically, and RNA integrity was monitored by denaturing formaldehyde/agarose gel electrophoresis. Total RNA (5 μg) was reverse-transcribed using oligo(dT) and Stratascript reverse transcriptase (Stratagene, La Jolla, CA). mRNA levels for mouse carbonic anhydrase (CA) IX, glucose transporter (GLUT) 1, erythropoietin, and PHD1, PHD2, and PHD3 were quantified with 2 μl of diluted cDNA reaction (corresponding to 1% of cDNA reaction) by real time RT-PCR. A SybrGreen qPCR reagent kit (Sigma) was used in combination with an MX3000P light cycler (Stratagene). Initial template concentrations of each sample were calculated by comparison with serial dilutions of a calibrated standard. To control for equal input levels, ribosomal protein S12 mRNA was determined, and data were expressed as ratios relative to S12 levels. Melting point analyses of amplified PCR products were performed after each run to verify specific amplification. For primer sequences see Supplemental Material.

Plasmid Constructions—Plasmids encoding full-length mouse HIF-1α, both wild-type and P402A and/or P563A mutants (note that all amino acid numbering corresponds to mouse HIF-1α sequences), were a kind gift of L. Poellinger (Stockholm, Sweden). A PCR product spanning the HIF-1α ODD region (amino acids 359–685) was cloned into the EcoRI site of pM3-VP16 (Clontech) to obtain one-hybrid constructs harboring an N-terminal GAL4 DNA-binding domain and a C-terminal VP16 transactivation domain. Resulting plasmids were termed G4.mHIF(359–685).VP16_wt, G4.mHIF(359–685).VP16_P402A, G4.mHIF(359–685).VP16_P563A, and G4.mHIF(359–685).VP16_PP/AA. For overexpression in mammalian cells, full-length human PHD2 and PHD3 (kindly provided by W. Kaelin, Jr., Boston, and I. Flamme, Wuppertal, Germany, respectively) were subcloned into pENTR4 and recombined into pcDNA3.1/nV5-DEST using Gateway technology (Invitrogen). Similarly, vectors for expression of GST-tagged PHD isoforms in Sf9 insect cells were constructed by recombining coding sequences for PHD2 and PHD3 into pDEST20 (Invitrogen). All primary cDNA inserts were sequenced (Microsynth, Balgach, Switzerland).

Transient Transfections—Cells were co-transfected with the indicated amounts of DNA using polyethyleneimine (Polysciences, Warrington, PA). Therefore, cells were grown on 100-mm dishes to subconfluency, and 200 μl of 150 mM NaCl containing a DNA/polyethyleneimine mixture (1:5, w/w) was added. For co-transfections, total DNA was kept constant by adding empty vector. Following overnight incubation, the cells were trypsinized and divided onto 12-well plates for luciferase assays and 100-mm dishes for immunoblotting.

Luciferase Assays—MEF-*Hif1a*$^{-/-}$ cells were transiently co-transfected with 2 μg of the indicated G4.mHIF(359–685).VP16 fusion constructs and up to 8 μg of the respective V5.PHD isoform, along with 1 μg of the GAL4-responsive reporter plasmid pGRE5xE1b, containing the firefly *luciferase* gene under control of E1b promoter and five GAL4 response elements (kind gift of D. Peet, Adelaide, Australia).

Identical amounts of total DNA were transfected in each experimental setting. Following transfection, cultures were grown for an additional 24 h at the indicated oxygen concentration and lysed in 100 μl of passive lysis buffer (Promega, Madison, WI). Luciferase reporter gene activity was determined in a microplate luminometer (Berthold, Regensdorf, Switzerland) using luciferase firefly substrate (Promega). Protein concentration in the lysates was determined by a Bradford assay, and relative luciferase activity was calculated from the ratio between relative light units and micrograms of protein.

RNA Interference—HeLa cells were plated at a density of 2×10^5 cells per single well of a 6-well plate. The day after, cells were transfected in fresh media with 80 nM siRNA duplexes targeting either human PHD2 (5′-ggacgaaagccaugguugcuuguua-3′, sense strand) or PHD3 (5′-gcuauccgggaaauggaacagguua-3′, sense strand) using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). After 4 h of culturing at 20% oxygen, cells were subjected to the indicated oxygen concentrations and grown for an additional period of 24 h prior to lysis.

Expression and Purification of PHDs—GST·PHD2 and GST·PHD3 were expressed in baculovirus-infected Sf9 insect cells according to the manufacturer's instructions (Invitrogen). After 80 h of infection, Sf9 cells were lysed in ice-cold 0.1% Nonidet P-40, 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 100 mM glycine, and 10 μM dithiothreitol. Crude lysate was cleared by centrifugation at 20,000 × *g* for 20 min, and supernatants were incubated with equilibrated glutathione-Sepharose beads (Amersham Biosciences) for 2 h at 4 °C with gentle agitation. Beads were washed three times with phosphate-buffered saline, and bound protein was eluted with 15 mM reduced glutathione, 50 mM Tris-HCl, pH 8.0, 5% glycerol, and 2 μM $FeSO_4$. Purity of recombinant proteins was estimated by SDS-PAGE and Coomassie Blue staining.

In Vitro Prolyl 4-Hydroxylation Assays—Enzymatic activity of recombinant PHD2 and PHD3 was determined by an *in vitro* hydroxylation assay performed essentially as described before (17, 21). Briefly, biotinylated mouse HIF-1α-derived peptides (amino acids 555–573, either wild-type or P563A mutant) were bound to NeutrAvidin-coated 96-well plates (Pierce). Hydroxylase reactions using purified recombinant GST·PHD2 or GST·PHD3 enzyme were carried out for 1 h at room temperature. A polycistronic expression vector for His_6- and thioredoxin-tagged pVHL/elongin B/elongin C (VBC) complex was kindly provided by S. Tan (Pennsylvania State University, University Park, PA). VBC was expressed in bacteria, purified by nickel affinity chromatography followed by ion exchange chromatography (Amersham Biosciences), and allowed to bind to the hydroxylated peptides. Bound VBC complex was detected by rabbit anti-thioredoxin antibodies and secondary horseradish peroxidase-coupled anti-rabbit antibodies (Sigma) using the 3,3′,5,5′-tetramethylbenzidine substrate kit (Pierce). The peroxidase reaction was stopped by adding H_2SO_4, and absorbance was determined at 450 nm in a microplate reader. For oxygen titration experiments, the assay was performed in the hypoxic glove box. All reagents and solutions were allowed to equilibrate to the indicated oxygen concentration. Inter-assay comparability was guaranteed by calibration of each experiment to an internal standard curve using hydroxyproline-containing peptides.

RESULTS

mRNA Levels of Inducible PHD Isoforms Show a Transient Maximum and Remain Up-regulated during Prolonged Hypoxia in Cell Culture—To study the kinetics of PHD expression during prolonged hypoxia (2% O_2 for up to 256 h), mRNA levels of all three PHDs were determined in MEF-*Hif1a*$^{+/+}$ and MEF-*Hif1a*$^{-/-}$ cells. PHD2 and PHD3 but not PHD1 mRNA levels were efficiently up-regulated under hypoxic conditions, peaked after 64–112 h, and remained elevated over the entire time course (Fig. 1*A*). As positive controls, mRNA levels of *GLUT1* and *CAIX*, two well established HIF-1 target genes, were measured in the same time course. Interestingly, hypoxic GLUT1 up-regulation followed a similar kinetics as PHD2 mRNA expression, whereas CAIX levels were maximally induced 48 h later, as observed for PHD3 (Fig. 1*A*). Because both PHDs were readily induced after 4 h, we investigated the early onset of induction in a shorter time course. Induction of PHD2 and PHD3 mRNA was detectable as early as 60–120 min after hypoxic stimulation (Fig. 1*B*). None of the PHD isoforms was induced in hypoxic MEF-*Hif1a*$^{-/-}$ cells, suggesting a non-redundant role of HIF-1α for hypoxic up-regulation of PHD mRNA in these cell lines. In addition, a lack of PHD2 and PHD3 mRNA induction was also observed in functionally HIF-1β/ARNT-deficient mouse Hepa1c4 but not wild-type Hepa1 hepatoma cells after 38 h of hypoxic stimulation (Fig. 1*C*). Thus, these genetically altered cellular models confirm HIF-1-dependent *PHD2* and *PHD3* gene expression in both acute and chronic hypoxia, which has been demonstrated previously mainly in siRNA experiments (12, 13, 22).

Tissue-dependent Hypoxic Induction of PHD2 and PHD3 mRNA in Mice—To date, no systematic investigation on hypoxic PHD mRNA induction *in vivo* was available. Thus, we assessed the grade of hypoxic PHD2 and PHD3 mRNA induction in acutely and chronically hypoxic mice.

To examine the effects of acute hypoxia, mice were treated for 4 h with an inspiratory gas mixture containing 0.1% carbon monoxide (CO), which induced a rapid onset of severe hypoxia by blocking ~50% of the oxygen-binding sites in hemoglobin (data not shown). Strong hypoxic induction of erythropoietin mRNA content in kidney as well as erythropoietin and GLUT1 mRNA in liver and brain confirmed the activation of the HIF system in these mice (Fig. 2). The various PHD mRNA isoforms showed a broad disparity of hypoxic activation; PHD1 was not markedly regulated by hypoxia in most tissues; PHD2 was widely induced by hypoxia, albeit at rather low levels; and PHD3 was strongly induced already following 4 h of tissue hypoxia in the lung (15.6-fold), liver (5.2-fold), and kidney (3.8-fold), but only moderate induction factors similar to PHD2 were observed in other organs. Of note, striated muscle tissue (heart and to some extent tongue) showed the highest normoxic expression values for the inducible PHD2 and PHD3 isoforms.

To investigate the kinetics of PHD mRNA induction under chronically hypoxic conditions, mice were exposed to

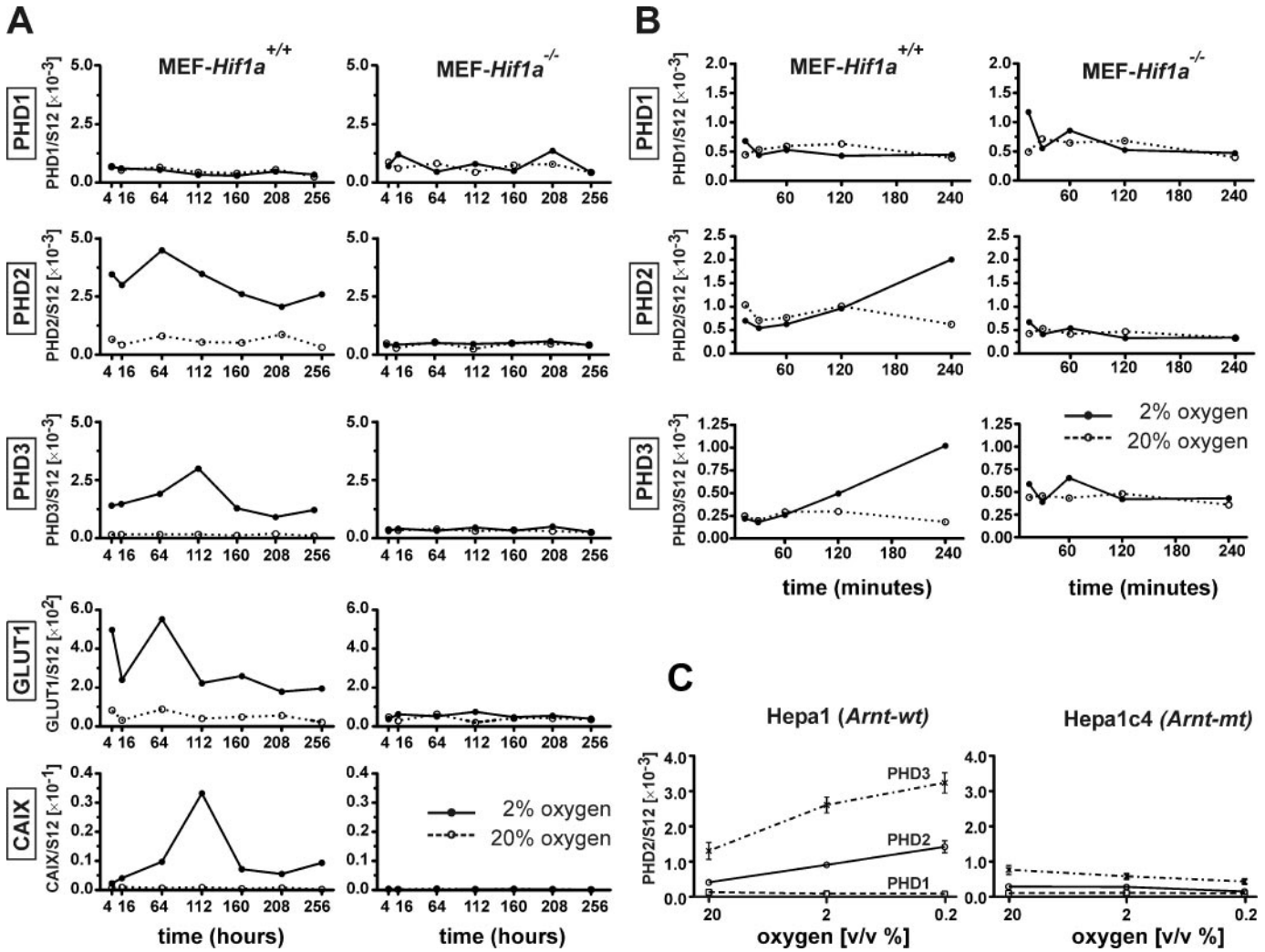

FIGURE 1. **HIF-1 target genes remain elevated during prolonged hypoxia.** MEF-*Hif1a*$^{+/+}$ and MEF-*Hif1a*$^{-/-}$ were cultured either at 20 or 2% oxygen for 4–256 h (*A*) or 15–240 min (*B*). mRNA levels for PHD1, PHD2, PHD3, GLUT1, and CAIX were quantified by real time RT-PCR and normalized to ribosomal protein S12 mRNA content. *C*, PHD1, PHD2, and PHD3 mRNA levels in Hepa1 *Arnt* wild-type (*wt*) and Hepa1c4 *Arnt* mutant (*mt*) cells cultured at 20, 2, or 0.2% oxygen for 38 h. Data are given as mean ± S.E. of $n = 3$ independent experiments.

inspiratory hypoxia (7.5% O_2) from 24 to 72 h, and mRNA levels were determined in liver, kidney, and brain. Although erythropoietin mRNA expression was induced to a similar extent in kidney and brain already after 24 h of hypoxia, erythropoietin mRNA in liver did not increase until 72 h of hypoxic exposure (Fig. 3*A*). Consistent with previous findings, serum levels of erythropoietin protein in these animals highly correlated with mRNA levels in kidney (Fig. 3*B*). Unexpectedly, the hypoxic inducibility of the known HIF target genes *GLUT1* and *CAIX* was rather small. Although strong hypoxic induction of both proteins in tumors and tumor-derived cell lines is well established, they were not induced in liver; only one of them (CAIX) was induced after prolonged hypoxia in kidney, and both of them showed a transient induction in brain (Fig. 3*C*). Nevertheless, these results confirmed the hypoxic status of the animals. Comparable results were obtained with the inducible PHD2 and PHD3 isoforms; only PHD2 showed a small, transient increase in the liver; only PHD3 was induced after prolonged hypoxia in the kidney; and both of them were transiently induced in the brain. Thus, under physiological inspiratory hypoxia, regulation of these genes appears to be more subtle than under *in vitro* conditions, in tumor hypoxia, or following severe tissue hypoxia in CO-treated mice.

Hypoxic Induction of PHD2 and PHD3 Proteins Is Accompanied by Decreased HIF-1α Protein Levels—Hypoxic up-regulation of PHD2 and PHD3 has the potential to affect protein abundance of HIF-1α during prolonged hypoxia. To further elaborate this hypothesis, we quantified protein levels of HIF-1α as well as PHD2 and PHD3 in different cell lines. As exemplarily shown for HEK293 cells, endogenous HIF-1α protein levels decreased concomitantly with the hypoxic increase of endogenous PHD2 and PHD3 levels when cells were cultured for up to 72 h at 1% O_2 (Fig. 4*A*). Similar results were obtained from lysates that were prepared under strictly hypoxic conditions, ruling out the possibility that increased levels of induced PHD enzymes might have influenced the protein levels of HIF-1α during lysis. HIF-1α mRNA levels in HEK293 were not significantly altered under the same hypoxic conditions, whereas PHD2 and PHD3 mRNAs were readily induced after

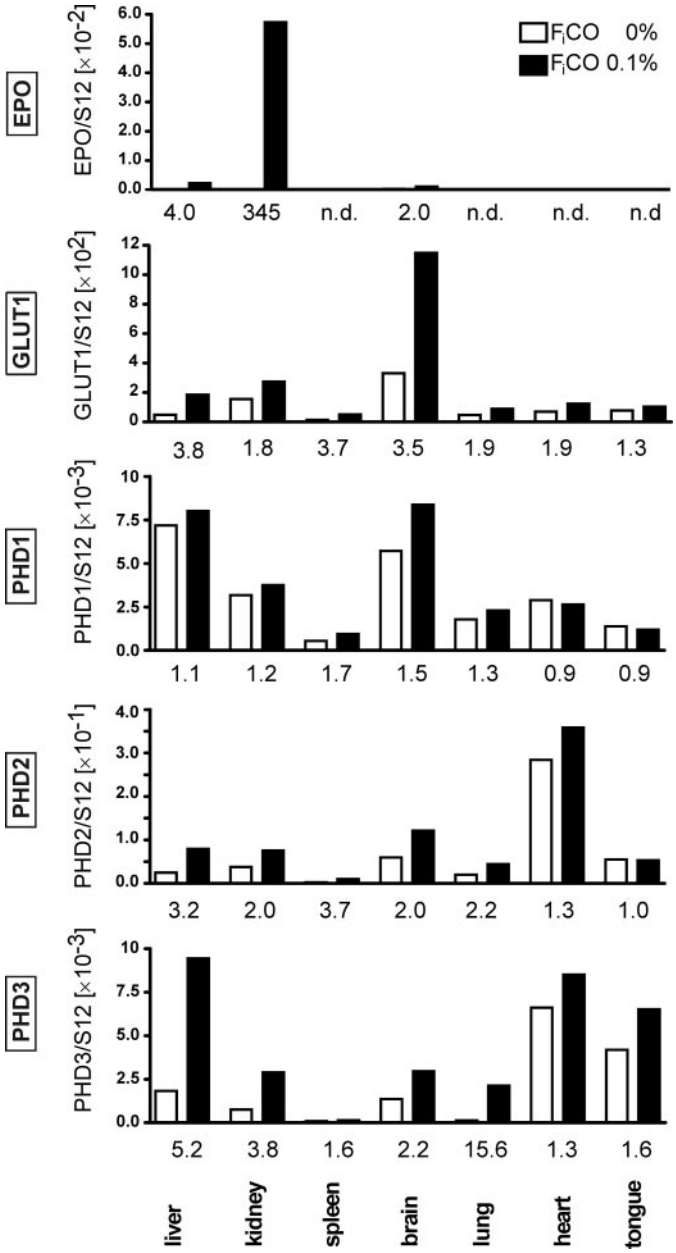

FIGURE 2. **Effects of tissue hypoxia on the inducibility of PHD isoforms *in vivo*.** Tissues were derived from mice kept either under normal air (F_iCO 0%) or in a gas mixture containing 0.1% CO (F_iCO 0.1%) for 4 h. PHD1, PHD2, PHD3, erythropoietin, and GLUT1 mRNA levels were determined by real time RT-PCR and normalized to S12 mRNA. Organ-specific hypoxic induction factors are indicated below the *bars*. Note the different scales (*n.d.*, not detectable).

4 h of hypoxia (Fig. 4*B*). Interestingly, hypoxic PHD3 mRNA induction was steadily induced during the entire hypoxic incubation, whereas PHD2 mRNA levels reached maximal induction already before 4 h of hypoxia and remained constant for up to 72 h of hypoxia.

A similar pattern of HIF-1α and PHD protein levels was observed in HeLa and Hep3B cells at oxygen concentrations as low as 0.2%, supporting the idea of a widespread regulative mechanism (see supplemental Fig. 1). These observations suggest that even under very limited oxygen supply, HIF-1α hydroxylation by PHDs functionally persists, implicating that HIF-1α turnover might be controlled by the PHD sensor system not only under normoxic conditions but also under severely hypoxic conditions.

A Second Hypoxic Insult Activates Another HIF-1α Response in Cells Adapted to Chronic Hypoxia—If the novel HIF-1α protein base line following adaptation to chronic hypoxia indeed resulted from an altered steady state of the HIF-PHD oxygen-sensing circuit, the adapted cells should react to a second, more severe hypoxic insult by acutely inducing HIF-1α to a similar extent as at the first hypoxic insult. To test this hypothesis, HEK293 cells were allowed to adapt to 1% oxygen for 72 h before they were exposed to 0.2% oxygen for up to 4 h. Although HIF-1α levels markedly decreased during chronic hypoxia, a rapid re-accumulation of HIF-1α protein was already observed 1 h after exposing the cells to 0.2% oxygen (Fig. 4*C*). A similar result was obtained when cells adapted to chronic hypoxia were treated with the PHD inhibitor dimethyloxalylglycine (DMOG), suggesting that PHDs are responsible for HIF-1α regulation even in hypoxically adapted cells (Fig. 4*D*).

PHD2 and PHD3 Silencing Increases Hypoxic HIF-1α Accumulation—As in HEK293 cells, simultaneous treatment of HeLa cells with hypoxia together with the PHD inhibitor DMOG led to additional accumulation of HIF-1α at oxygen concentrations as low as 0.2% (Fig. 5*A*). To provide further evidence that endogenous PHDs control HIF-1α protein levels even under hypoxic conditions, we applied siRNA to knock down PHD2 and PHD3 in HeLa cells. Interestingly, silencing of either PHD2 or PHD3 equally increased HIF-1α levels, irrespective of whether the cells were cultured at 20 or 1% oxygen (Fig. 5*B*). Although apparently both isoforms are involved in regulating HIF-α stability over a broad range of oxygen concentrations, combined silencing of PHD2 together with PHD3 most efficiently up-regulated HIF-1α at 20% as well as 1% O_2 (Fig. 5*B*).

PHDs Retain Functional HIF-1α Degradation Activity Even under Severely Hypoxic Conditions in Cell Culture—Current knowledge about oxygen substrate requirements of the PHD-dependent hydroxylation reaction is exclusively based on data derived from biochemical *in vitro* studies using purified enzymes and short peptides containing one single HIF-1α hydroxylation site as substrates. To further examine the activity of PHD enzymes under lowered oxygen availability in a cellular context, a luciferase-based mammalian one-hybrid approach to quantify HIF-1α stability in a feedback-uncoupled cellular system was established. A series of mammalian expression vectors coding for the mouse HIF-1α ODD domain, including mutant forms of the hydroxylation sites (P402A and/or P563A), were N-terminally fused to the yeast GAL4 DNA-binding domain and C-terminally fused to the herpes simplex virus VP16 transactivation domain (Fig. 6*A*). The expressed fusion proteins cannot transactivate endogenous HIF target genes, including *PHD2* and *PHD3*, but activate a co-transfected GAL4-responsive reporter gene. The use of HIF-1α-deficient MEF-*Hif1a*$^{-/-}$ cells ensures very low basal levels of endogenous PHDs (Fig. 1*A*) and avoids confusion with hypoxic up-regulation of endogenous PHD2 and PHD3 by endogenous HIF-1, allowing us to experimentally define the PHD levels by transfection of

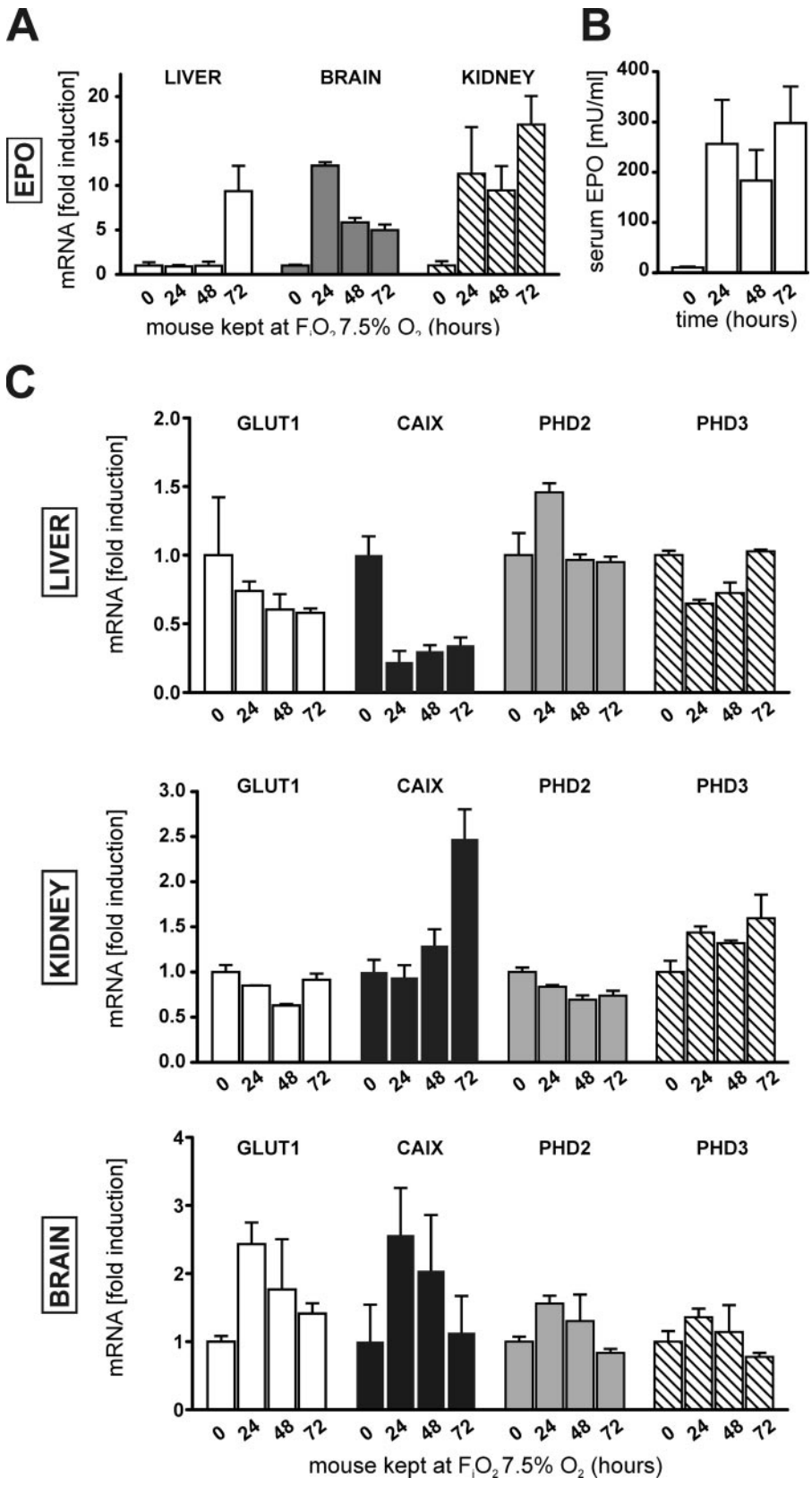

FIGURE 3. **Effects of inspiratory hypoxia on HIF target gene expression *in vivo*.** Mice were kept at an F_iO_2 of 20% O_2 (0 h of hypoxia) or 7.5% O_2 for up to 72 h. Erythropoietin mRNA levels in liver, brain, and kidney were quantified by real time RT-PCR (*A*), and corresponding serum levels of erythropoietin were measured by radioimmunoassay (*B*). *C*, GLUT1, CAIX, PHD2, and PHD3 mRNA levels were measured in all three tissues. All mRNA data were normalized to S12 mRNA levels and are expressed as hypoxic induction factors. *Bars* represent the mean ± S.E. of $n = 3$ mice per group.

expression vectors. Because the HIF-1α-ODD confers oxygen-dependent instability to the fusion protein, luciferase reporter gene activity directly reflects protein stability.

First, we tested the relative importance of the two PHD target proline residues within the mouse HIF-1α-ODD residues 359–685. Although reporter gene activation of the wild-type and single proline mutant fusion proteins was similarly reduced by PHD2 overexpression, mutation of both prolines was necessary to render the HIF-1α-ODD insensitive to PHD2 overexpression (Fig. 6*B*). Although similar effects of PHD overexpression on ODD stability have been reported previously only for normoxic conditions (23), our data show functional relevance of both hydroxylation sites also for hypoxic (2% O_2) regulation of HIF-1α protein levels (Fig. 6*B*).

In the next step, both oxygen concentration and PHD protein abundance were titered simultaneously in co-transfection experiments. Interestingly, co-expression of low amounts of PHD2, which were not yet detectable by immunoblotting, already reduced HIF-1α-ODD stability to a minimum, which was not further lowered by increased amounts of PHD2 (Fig. 6*C*). Reducing the oxygen concentration to 4% or below revealed a successive decrease of hydroxylase activity at a given amount of PHD2 enzyme. However, increasing the abundance of PHD2 by stepwise duplication of the amount of transfected PHD2 expression vector again demonstrated that induced PHD levels can compensate for reduced oxygen availability. Indeed, increased PHD2 compensated for oxygen concentrations as low as 0.2% (Fig. 6*C*).

Similar results were obtained by PHD3 overexpression, which also resulted in decreased HIF-1α-ODD stability under normoxic and moderately hypoxic conditions (Fig. 6*D*). However, forced expression of PHD3 failed to further decrease HIF-1α-ODD stability at 0.2% oxygen, suggesting a principal differ-

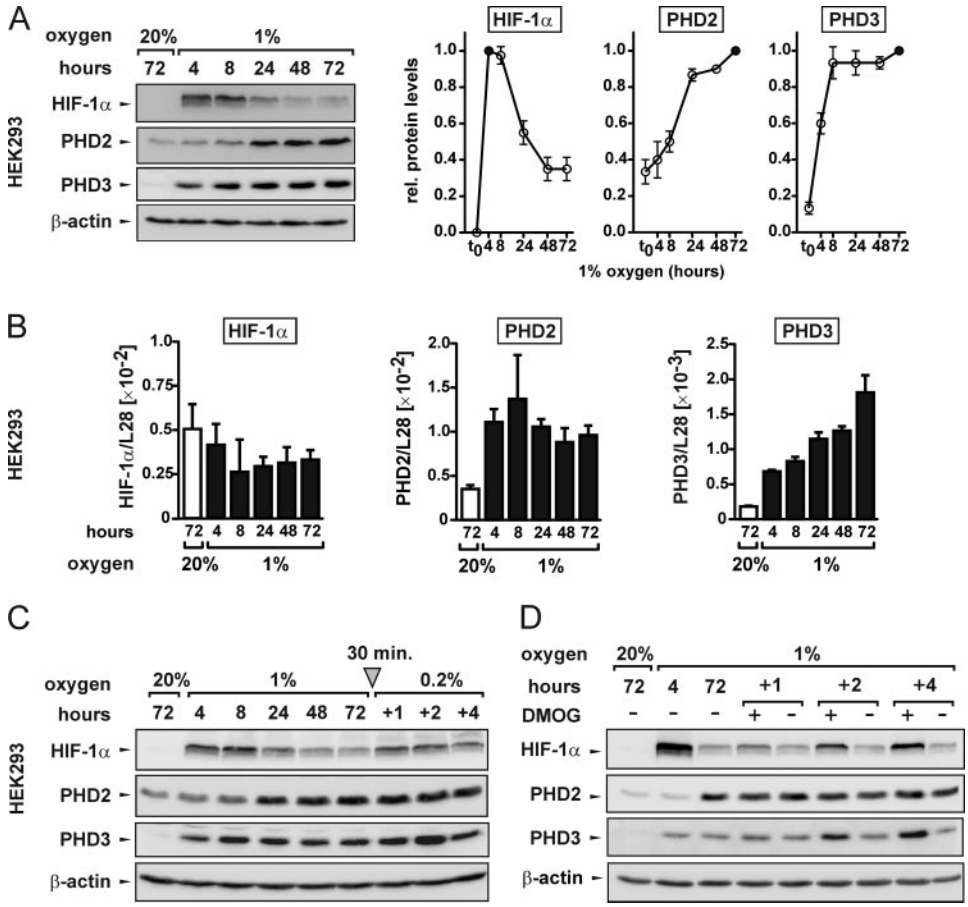

FIGURE 4. **Decreased HIF-1α protein levels parallel increased PHD2 and PHD3 under chronically hypoxic conditions.** *A,* HEK293 cells were cultured at 20 or 1% oxygen for up to 72 h. HIF-1α, PHD2, PHD3, and β-actin (loading control) protein levels were estimated by immunoblotting of total cell lysates. A representative experiment is shown along with densitometric quantification of bands for $n = 3$ (PHDs) or $n = 4$ (HIF-1α) independent experiments. For inter-assay normalization, time points expressing highest protein levels were arbitrarily defined as 1 (*filled circles*; mean ± S.E.). *B,* HIF-1α, PHD2, and PHD3 mRNA levels in HEK293 cells were quantified by real time RT-PCR and normalized to ribosomal protein L28 mRNA. *Bars* represent mean ± S.E. of triplicate experiments. *C,* HEK293 cells were exposed to 1% oxygen for 72 h and subjected to 0.2% oxygen for additional 1–4 h without reoxygenation. Lowering the oxygen concentration from 1 to 0.2% took ~30 min. Immunoblotting was performed as in *A*. *D,* HEK293 cells were grown as in *C* and treated with a PHD inhibitor ("+", 1 mM DMOG) or solvent alone ("−", 0.1% Me_2SO) for 1–4 h at 1% O_2. Immunoblotting was performed as described in *A*.

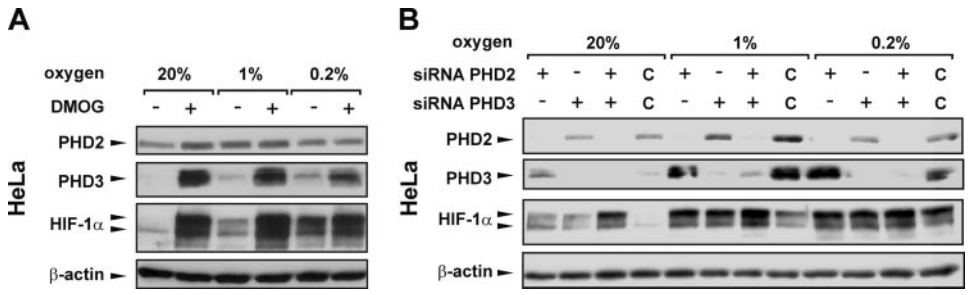

FIGURE 5. **Endogenous PHD2 and PHD3 enzymes control HIF-1α accumulation under normoxic as well as under hypoxic conditions.** *A,* HeLa cultures were subjected to 20, 1, or 0.2% oxygen for 4 h and simultaneously treated with a PHD inhibitor (+, 1 mM DMOG). PHD2, PHD3, HIF-1α, and β-actin protein levels were determined by immunoblotting of total cell lysates. *B,* HeLa cells were transiently transfected with siRNAs directed against either PHD2, PHD3, or a combination of both and were cultured at the indicated oxygen concentrations for 24 h. Transfection of cells with a similarly constructed but unspecific siRNA oligonucleotide served as negative control ("*C*"). Immunoblotting was performed as described above.

ence in oxygen-dependent hydroxylase activities between PHD2 and PHD3 under various degrees of hypoxia *in vivo* (Fig. 6*D*).

Purified PHDs Retain Functional Hydroxylation Activity Even under Severe Hypoxic Conditions in a Cell-free System in Vitro—The differences in hydroxylase activity of PHD2 and PHD3 under severe hypoxia described above prompted us to investigate the activity of PHDs using a cell-free *in vitro* approach. Therefore, GST-tagged PHD2 and PHD3 were purified from baculovirus-infected Sf9 insect cells (Fig. 7*A*). These enzymes were used to hydroxylate a mouse HIF-1α-ODD-derived peptide coupled to 96-well plates, and binding of a purified VBC complex to the hydroxylated peptide was measured by enzyme-linked immunosorbent assay. Each PHD preparation was diluted to obtain equal VBC binding after 1 h of hydroxylation (arbitrarily defined as "1-fold input"). These enzyme concentrations were subsequently increased to mimic the hypoxic induction of PHDs *in vivo*. Under normoxic conditions, both PHDs showed a similar increase in activity with increasing protein amounts (Fig. 7*A*). Peptide hydroxylation was within the linear range of the assay over the time frame of experiments, even at highest enzyme concentrations (data not shown).

When the assay was performed at an oxygen concentration of 2%, PHD2 hydroxylation activity was only about half of the normoxic activity after 1 h of reaction (hydroxyproline contents of 10.1 ± 0.2% at 20% O_2 and 5.7 ± 0.5% at 2% O_2; $n = 3$, mean ± S.D.; 8-fold enzyme input). However, HIF-1α-ODD peptide hydroxylation by PHD3 was only slightly decreased under these conditions (10.3 ± 0.2% at 20% O_2 and 8.2 ± 1.1% at 2% O_2; $n = 3$, mean ± S.D.; 8-fold enzyme input). Further reduction in oxygen availability decreased proline hydroxylation, but significant hydroxylation could still be observed at oxygen concentrations as low as 0.2%. Indeed, an 8-fold protein input of PHD2 or PHD3 resulted in similar peptide hydroxylation at 0.2% O_2 as a 4-fold input at 0.5% O_2 or a 2-fold input at 1% O_2, suggesting that increased PHDs can compen-

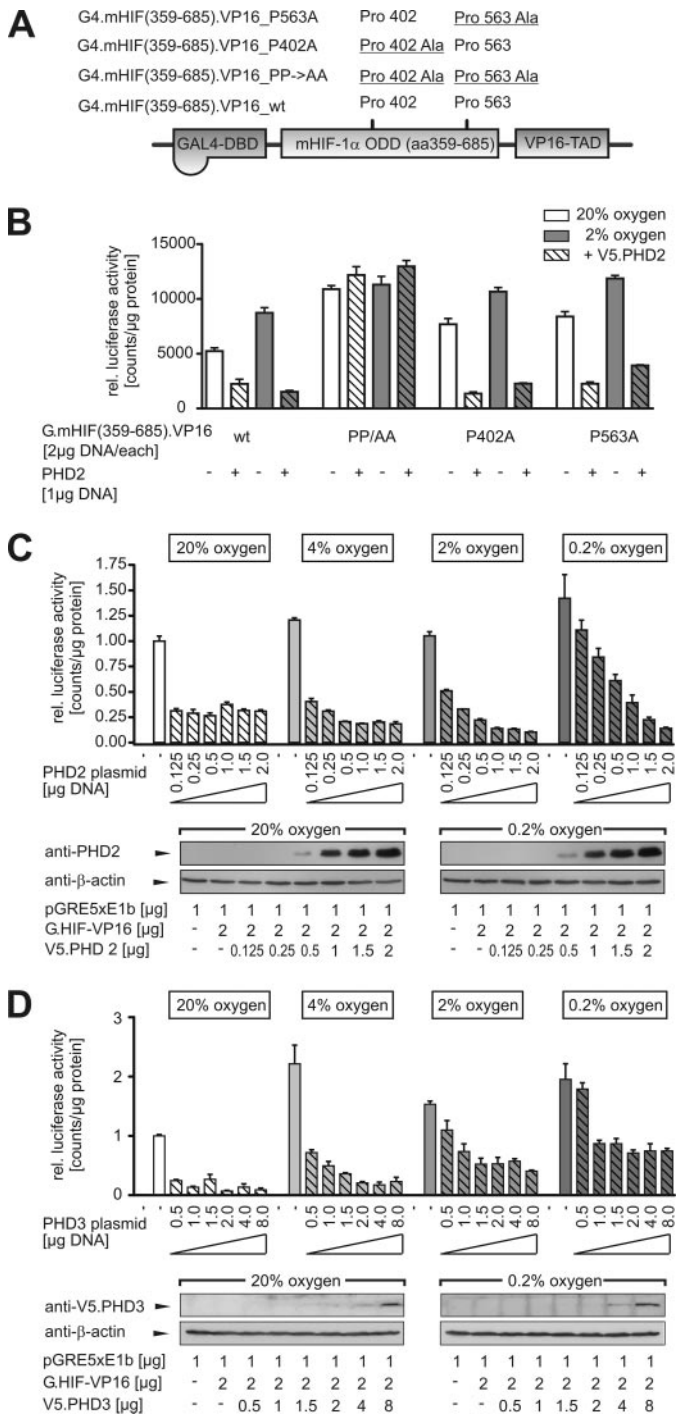

FIGURE 6. **PHD2 and PHD3 retain hydroxylase activity at low oxygen concentrations.** *A,* oxygen-sensitive one-hybrid fusion constructs. Mutations of Pro-402 and Pro-563 are *underlined*. *B,* MEF-*Hif1a*$^{-/-}$ cells were co-transfected with 2 μg of one-hybrid construct, 1 μg of a GAL4-responsive reporter gene, and 1 μg of V5.PHD2 or empty expression vector. Luciferase activity was measured 24 h after incubation at 20 or 2% oxygen, and relative light counts were normalized to total protein content in the lysates. Likewise, the indicated amounts of V5.PHD2 (*C*) and V5.PHD3 (*D*) were co-transfected with 2 μg of the wild-type (*wt*) one-hybrid construct and 1 μg of the reporter gene. Aliquots of transfected cells were then cultured simultaneously at various oxygen concentrations, and luciferase activities were determined after 24 h as described above. In addition, lysates were immunoblotted to verify expression of exogenous V5.PHD isoforms (*bottom panels*). Representative experiments performed in triplicate experiments are shown as means ± S.E.

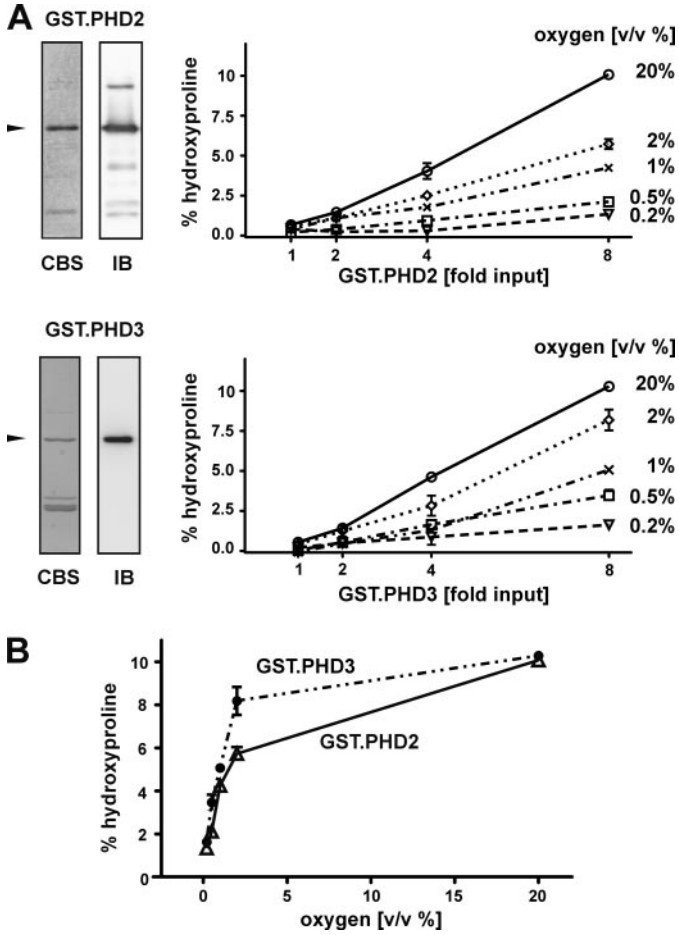

FIGURE 7. **Recombinant GST·PHD2 and GST·PHD3 proteins are active under hypoxic conditions *in vitro*.** *A,* GST.PHD2 and GST.PHD3 were purified from baculovirus-infected Sf9 insect cells, and their purity was checked by SDS-PAGE and Coomassie Blue staining (*CBS*) or by immunoblotting (*IB*) using polyclonal anti-PHD2 or anti-PHD3 antibodies. *In vitro* hydroxylation reactions were performed at various oxygen concentrations with increasing amounts of PHD2 and PHD3 enzymes, and hydroxyproline content was estimated from a calibration curve. *B,* oxygen dependence of hydroxylation is shown for 8-fold enzyme input. Enzyme activity is given as percentage of hydroxyproline after 1 h of reaction. All values are in the linear range of the assay and are shown as mean values ± S.E. of triplicate experiments.

sate for decreased oxygen also *in vitro* (Fig. 7*A*). Moreover, both enzymes showed a strikingly linear increase of activity for the range of 0.2 to 2% oxygen, thus fulfilling one of the most important criterions for a cellular oxygen sensor at physiologically relevant tissue O_2 concentrations (Fig. 7*B*).

DISCUSSION

The existence of a functional feedback loop limiting the hypoxic response has been suspected already when HIF was cloned, because nuclear HIF-1α declined despite ongoing hypoxia (24). Similar observations were then reported by a multitude of other groups. However, the underlying mechanism remained unknown. We previously reported that forced expression of HIF-1α followed a similar feedback kinetics, but in this case under normally oxygenated conditions (25). This observation suggested that HIF itself, rather than the hypoxic stimulus, is required for the feedback regulation. One such mechanism might be HIF-dependent induction of pVHL by "late hypoxia" (26). However, other studies could not find any

induction of pVHL under hypoxic conditions (27, 28), and we did not observe any oxygen- or HIF-1α-dependent difference in pVHL mRNA levels during the 256-h time course described in this study (data not shown).

The identification of the PHD oxygen sensors shed new light on the process of HIF feedback control, as it became clear that *PHD2* and *PHD3* are hypoxia-inducible HIF target genes (2, 9–13, 29). It has been postulated that this PHD up-regulation confers accelerated degradation of HIFα upon re-oxygenation (9, 12, 22). Indeed, re-oxygenation from 1 to 20.9% (air) oxygen after 18 h decreased the half-life of HIF-1α when compared with similarly treated cells that were exposed to hypoxia for only 1 h (12).

However, physiological tissue pO_2 corresponds to ~2–5% oxygen concentrations in air rather than the widely used 20.9% normoxic oxygen concentration. Even though the pericellular pO_2 is lower at the bottom of unstirred tissue culture dishes (30), standard cell culture conditions are to be considered "hyperoxic," which reflects an unphysiological condition to which PHDs are not normally exposed. Rather, PHDs *in vivo* are constantly functioning under pO_2 levels far below their *in vitro* K_m values (14). These considerations suggested a PHD-HIF feedback loop that might be active even under chronically low tissue oxygenation.

In line with this hypothesis, we observed undulating HIF-1α-dependent mRNA levels of CAIX, GLUT1, PHD2, and PHD3 in MEF cells, and we detected tissue-specific variations in hypoxically induced PHD2 and PHD3 mRNA levels in mice *in vivo*. So far, PHD expression levels were only known from established tumor cell lines or normoxic tissues (9, 10, 22, 31–33). We found an impressive up-regulation of PHD3 mRNA levels in hypoxic lung tissue, confirming previous reports for lung cancer-derived A549 cells (9, 28). Similarly, the observed induction factors in liver were roughly the same as determined in Hepa1 hepatoma cells used in this study.

We then concentrated on three major organ systems that are particularly sensitive to hypoxia and hence involved in the pathophysiology of shock. Interestingly, although the examined HIF target genes were all induced in the brain, different kinetics for GLUT1, CAIX, PHD2, and PHD3 were observed in kidney and liver. Expression levels of CAIX were highly increased in the kidney after 72 h of hypoxia, whereas a reverse effect was observed in liver. Because the kidney, besides the lung, is the major organ involved in systemic pH regulation, these results suggest a physiological role for CAIX in pH maintenance during hypoxia, as has been proposed for solid tumors (34). Regarding PHD mRNA induction, temporal up-regulation of at least one isoform was observed in each of the three tissues in hypoxic animals, confirming that PHD up-regulation indeed occurs in a hypoxic organism, although the extent of induction is tissue-specific. Not surprisingly, decreasing the oxygen transport capacity in mice by 0.1% CO inspiration was much more efficient in up-regulation of HIF target genes than inspiratory hypoxia itself, suggesting that adaptational mechanisms, such as increased heart rate, depth of breath, and reduced oxygen consumption compensated for decreased inspiratory O_2 concentration when oxygen transport is intact. We have shown previously that temporal and spatial accumulation of HIF-1α *in vivo* greatly differs, even in neighboring cells, suggesting that factors other than oxygen availability affect the PHD-HIF system (35). In fact, we recently found that the FK506-binding protein FKBP38 regulates specifically the abundance of PHD2.[3]

Whatever regulates cellular PHD levels, functional PHD induction requires that these enzymes must be active under a broad range of physiologically relevant conditions. In order to experimentally support this hypothesis, we used a HIF-1α-ODD reporter construct in a HIF-1α negative cell line to breach the suggested gene activation-protein degradation loop. The HIF-1α-ODD confers normoxic degradation to the resulting hybrid fusion protein (36), and similar constructs have been used lately as bioluminescent reporter systems monitoring the state of tissue oxygenation *in vivo* (37). Our experiments revealed that PHD-dependent fusion-protein destabilization was effective at oxygen concentrations as low as 0.2% O_2 and that increasing the amount of enzyme partially compensated for reduced oxygen availability. These data are consistent with a recent work (38) employing hydroxylation-specific antibodies that showed persistent HIF-1α hydroxylation at equally low oxygen concentrations.

Although we observed a comparable oxygen dependence between PHD2 and PHD3 purified from Sf9 insect cells *in vitro*, the ability of PHD3 to compensate for low oxygen concentrations in the cellular model was consistently lower than that of PHD2 (compare Fig. 6, *C* and *D*). In line with the former observation, *in vitro* transcribed and translated PHD2 and PHD3 enzymes have been shown to be equally active in another study (39). Thus, isoform-specific regulation of distinct PHDs *in vivo* is likely to depend on critical co-factors that might be depleted in purified enzyme preparations or become limited when PHDs are overexpressed. Supporting this notion, PHD3 has recently been described to serve as a substrate of the eukaryotic chaperonin complex TRiC that might be required for appropriate folding and full enzymatic activity (40).

Another recent work demonstrated increased binding of PHD3 to HIF-1α in the presence of OS-9 (41). As suggested by these authors, PHDs form functional multiprotein complexes that could affect binding affinity and target sequence selectivity in a cellular context. However, binding of OS-9 to human HIF-1α necessarily requires amino acids 692–785 that were neither present in the peptide used for the hydroxylation assays nor in the one-hybrid construct. Thus, PHD3 activity rather than binding might be attenuated in our cellular model system. Of note, protein degradation of PHD3 (and PHD1) has been shown to be enhanced under hypoxia by a mechanism involving the ubiquitin ligase Siah2, adding yet another layer of regulation on the HIF-PHD system (42).

Also, binding of PHD2 to HIF-1α has been suggested to repress the *N*-transactivation domain activity (43). Interestingly, we observed residual binding of recombinant PHD2 to immobilized HIF-1α ODD peptides *in vitro*, suggesting a repressive function of PHD2 particularly on truncated HIF-1α (data not shown). Although these effects were rather small compared with the HIF-1α-destabilizing function of enzymatic

[3] S. Barth, R. Wirthner, R. H. Wenger, and G. Camenisch, manuscript in preparation.

PHD activity, they still could have partially influenced the transcriptional activity of the fusion constructs used in our study.

In conclusion, the present work demonstrates that an essential pre-requirement for a PHD abundance-dependent modulation of the oxygen-sensing system is fulfilled; PHDs are operative under a wide variety of even severely hypoxic oxygen concentrations. These findings led to the intriguing suggestion of a flexible oxygen threshold of the PHD-HIF system that steadily adapts to altered tissue oxygenation.

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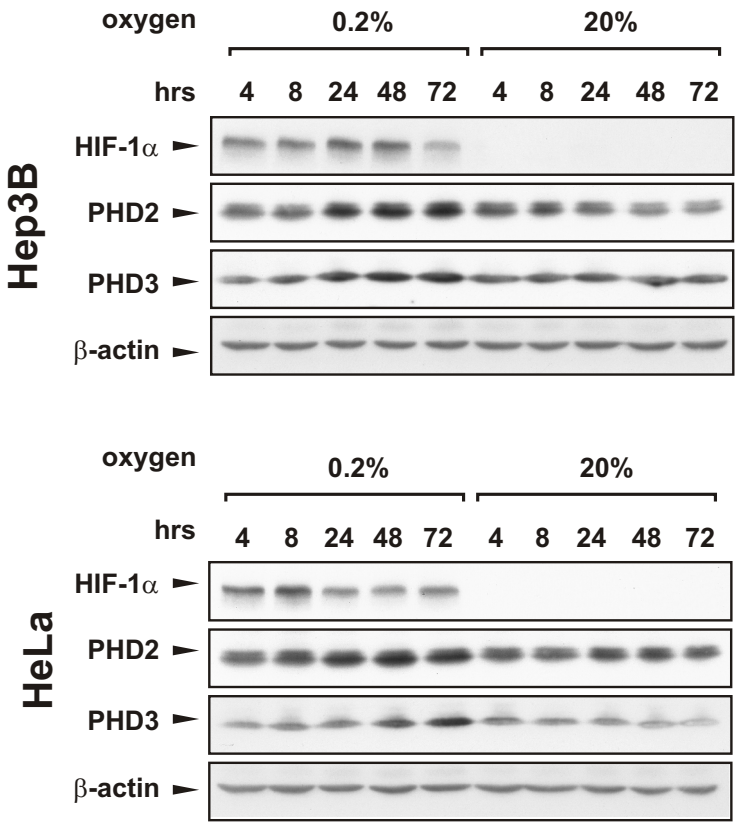
gene	species	Genbank Accession No.	forward primer	reverse primer
CAIX	mouse	AJ245857	5'- gctgtcccatttggaagaaa -3'	5'- ggaaggaagcctcaatcgtt -3'
GLUT1	mouse	M23384	5'- tctctgtcggcctctttgtt -3'	5'- gcagaagggcaacaggatac -3'
EPO	mouse	NM_007942	5'- atctgcgacagtcgagttct -3'	5'- gtatccactgtgagtgttcg -3'
PHD1	mouse	NM_053208	5'- ttgcctgggtagaaggtcac -3'	5'- gctcgatgttggctaccact -3'
PHD2	mouse	NM_053207	5'- gcaacggaacaggctatgtc -3'	5'- ctcgctcatctgcatcaaaa -3'
PHD3	mouse	NM_028133	5'- caacttcctcctgtccctca -3'	5'- ggctggacttcatgtggatt -3'
S12	mouse	NM_011295	5'- gaagctgccaaagccttaga -3'	5'- aactgcaaccaaccaccttc -3'
PHD2	human	NM_022051	5'- gaaagccatggttgcttgtt -3'	5'- ttgccttctggaaaaattcg -3'
PHD3	human	NM_022073	5'- atcgacaggctggtcctcta -3'	5'- cttggcatcccaattcttgt -3'
HIF-1α	human	NM_001530	5'- tccgatggaagcactagaca -3'	5'- tggtgacaactgatcgaagg -3'
L28	human	NM_000991	5'- gcaattccttccgctacaac -3'	5'- tgttcttgcggatcatgtgt -3'

Supplemental Table 1



SUPPLEMENTAL FIGURE 1. **Reciprocal regulation of HIF-1α and PHD protein levels during prolonged hypoxic culturing.** Hep3B- and HeLa cells were cultured at 20% or 0.2% oxygen for up to 72 hours. HIF-1α, PHD2, PHD3 and β-actin (loading control) protein levels were determined by immunoblotting of total cell lysates (75 μg total protein).

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Forum Original Research Communication

Regulated Function of the Prolyl-4-Hydroxylase Domain (PHD) Oxygen Sensor Proteins

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ABSTRACT

Cellular oxygen is sensed by prolyl-4-hydroxylase domain (PHD) proteins that hydroxylate hypoxia-inducible factor (HIF) α subunits. Under normoxic conditions, hydroxylated HIFα is bound by the von Hippel–Lindau (pVHL) tumor suppressor, leading to ubiquitinylation and proteasomal degradation. Under hypoxic conditions, hydroxylation becomes reduced, leading to HIFα stabilization. The authors recently showed that changes in PHD abundance and activity can regulate HIFα stability under normoxic as well as under hypoxic conditions. Thus, the PHD oxygen sensors themselves represent effectors of cellular signalling pathways as well as potential drug targets. Here, a cell-free *in vitro* microtiter plate-based peptide hydroxylation assay was used to investigate the influence of ferrous iron, Krebs cycle intermediates, transition metals, and vitamin C and other antioxidants on the activity of purified PHD1 to 3. PHD activity depends not only on oxygen availability but is also regulated by iron, vitamin C, and Krebs cycle intermediates, suggesting a physiological relevance of their cellular concentrations. Copper but not iron, cobalt, or nickel salts catalyzed vitamin C oxidation. While vitamin C is essential for PHD activity *in vitro*, *N*-acetyl-L-cysteine had no effect, and gallic acid or *n*-propyl gallate efficiently inhibited the activity of all three PHDs, demonstrating different functions of these antioxidants. *Antioxid. Redox Signal.* 9, 1329–1338.

INTRODUCTION

Oxygen availability affects many physiological and pathophysiological processes, including embryonic development, adaptation to high altitudes, wound healing, inflammation, cancer, and ischemic diseases such as infarction and stroke. Central to the understanding of these processes is the elucidation of the molecular mechanisms by which cells react and adapt to insufficient oxygen supply (hypoxia). Oxygen availability is measured by a family of oxygen-dependent protein hydroxylases that regulate the abundance and activity of hypoxia-inducible transcription factor (HIF) α subunits (47). HIFs in turn control the expression levels of effector genes involved in either anticipatory metabolic changes, adaptive survival, or programmed death of the affected tissue (58). HIFs are heterodimeric transcription factors consisting of one out of three different oxygen-sensitive HIFα subunits (HIF-1α, HIF-2α, or HIF-3α) and a common constitutive HIFβ subunit. While HIF-1 and HIF-2 $\alpha\beta$ heterodimers function as transcriptional activators of oxygen-regulated target genes, the role of HIF-3α is less clear and a short splice variant of HIF-3α, termed inhibitory PAS protein (IPAS), functions as a transcriptional repressor (57).

Dependent upon the cellular oxygen partial pressure (pO_2), a family of prolyl-4-hydroxylase domain (PHD) enzymes covalently modify two proline residues within the oxygen-dependent degradation (ODD) domain of HIFα subunits. The PHD family is comprised of three members called PHD1,

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PHD2, PHD3, or HIF prolyl hydroxylase (HPH) HPH3, HPH2, HPH1, respectively (7, 14). A fourth member, called PH4, regulates HIFα under overexpression conditions only (40). Upon hydroxylation under normoxic conditions, HIFα is bound by the von Hippel–Lindau (VHL) tumor suppressor protein and targeted for proteasomal destruction (35). Thus, the high turnover rate of HIFα subunits allows for an instantaneous stabilization under hypoxic conditions (21). According to the current model, also the asparagine hydroxylase function of the factor inhibiting HIF (FIH) becomes impaired when oxygen availability is further decreased, resulting in a decrease in C-terminal HIFα asparagine hydroxylation (44). This allows for the progressively increased recruitment of p300/CPB transcriptional co-activators, leading to a successively higher transcriptional function of HIF (28, 31).

While all three PHDs can hydroxylate HIFα with similar efficiency, PHD2 has been suggested to play the main role for normoxic HIFα turnover (6). Consistent with these *in vitro* findings, PHD2 but not PHD1 or PHD3 knock-out mice die during embryonic development (51). The three PHDs are expressed in most organs, but there are strikingly high levels of PHD3 mRNA in the heart and of PHD1 mRNA in the testis (50). In addition to HIFα, there is some evidence that the iron regulatory protein IRP2, the RNApol II large subunit Rpb1, the heme synthesis enzyme ALAS2, and IκB kinase-β are regulated by PHDs (1, 10, 27, 54, 55). Of note, these experiments were mainly based on pharmacological inhibition of PHDs and no direct evidence for protein hydroxylation has been provided yet. Interestingly, ankyrin repeats within the NF-κB family (p105) and IκBα were shown to be efficiently hydroxylated by FIH (9). The function of this FIH-dependent hydroxylation, however, is unclear up to date.

The regulation of PHD expression and activity has become of considerable interest in the recent past. Endogenous tricarboxylic acid cycle intermediates and reactive oxygen species (ROS) have been reported to inhibit PHD function and hence link mitochondrial function with PHD-dependent oxygen sensing (12, 15, 43, 48). Small molecule inhibitors that can be added exogenously are currently being developed for clinical tissue protection in diseases associated with oxygen deprivation. However, little is known on the differential regulation of the three PHD family members. We therefore set out to investigate the control of each PHD family member individually by small molecules as well as by newly identified protein interactors and by HIF-dependent feedback regulation.

MATERIALS AND METHODS

Expression and purification of PHDs

GST-PHD1, GST-PHD2, and GST-PHD3 were expressed in baculovirus-infected Sf9 insect cells and purified as described before (50). Briefly, after 80–110 h of infection, Sf9 cells were lysed in ice-cold 0.1% NP-40, 10 m*M* Tris-HCl pH 7.5, 100 m*M* NaCl, 100 m*M* glycine, and 10 μM DTT. Cleared lysates were incubated with equilibrated glutathione-sepharose beads (Amersham, Dübendorf, Switzerland) for 2 h at 4°C with gentle agitation. After washing of the beads three times with PBS, bound protein was eluted with 15 m*M* reduced glutathione, 50 m*M* Tris-HCl pH 8.0, and 2 μM $FeSO_4$. Purity of recombinant fusion-proteins was routinely estimated by SDS-PAGE and coomassie blue staining.

In vitro *prolyl-4-hydroxylation assays*

Enzymatic activity of recombinant PHDs was determined as described before (32, 41). Biotinylated peptides (100 ng/well) derived from human HIF-1α aa 556 to 574 (either wild-type or P564A mutant) were bound to NeutrAvidin-coated 96-well plates (Pierce, Perbio, Lausanne, Switzerland). Purified recombinant PHD enzymes were used to hydroxylate the peptides in the presence of 0.5 m*M* 2-oxoglutarate, 2 m*M* ascorbate, 10 μM $FeSO_4$ in 20 m*M* Tris-HCl pH 7.5, 5 m*M* KCl, 1.5 m*M* $MgCl_2$ for 1 h at room temperature. A polycistronic expression vector for His_6- and thioredoxin-tagged pVHL/elongin B/elongin C (VBC) complex was kindly provided by S. Tan (Pennsylvania, PA). VBC was expressed in bacteria, purified by nickel affinity chromatography, followed by ion exchange chromatography and buffer exchange gelfiltration. VBC complex was allowed to bind to the hydroxylated peptides and bound VBC complex was detected by rabbit anti-thioredoxin antibodies, followed by secondary horseradish peroxidase-coupled anti-rabbit antibodies (Sigma, Buchs, Switzerland), using the TMB (3,3′,5,5′-tetramethylbenzidine) substrate kit (Pierce). The peroxidase reaction was stopped by adding H_2SO_4 to 1 *M* and absorbance was determined at 450 nm in a microplate reader.

Ascorbate oxidation assays

Ascorbate oxidation to dehydroascorbate was measured spectrophotometrically as described (49). Therefore, the decrease in absorbance at 265 nm was measured in a 100 μM ascorbate solution in 20 m*M* Tris-HCl pH 7.5, 5 m*M* KCl, 1.5 m*M* $MgCl_2$ using open cuvettes with free access to air.

Hypoxia reporter cells

The Chinese hamster ovary (CHO) cell line stably transfected with a hypoxia-responsive firefly luciferase reporter gene (termed HRCHO5) has been described before (56). Cell lysis and determination of luciferase activity was performed according to the manufacturer's instructions (Promega, Wallisellen, Switzerland). Relative light units were measured in a 96-well luminometer (Berthold, Regensdorf, Switzerland) and normalized to the protein concentration determined by the Bradford assay (Biorad, Reinach, Switzerland).

RESULTS

Ferrous iron availability is essential for the function of all three PHDs

GST-tagged PHD1, PHD2, and PHD3 were purified from Sf9 insect cells to 80–90% purity and their activities were analyzed by a VBC binding assay. All three PHDs induced VBC binding to the wild-type P564 but not to the mutant P564A-containing peptide derived from the HIF-1α ODD (Fig. 1A). The PHD preparations were calibrated with hydroxyproline

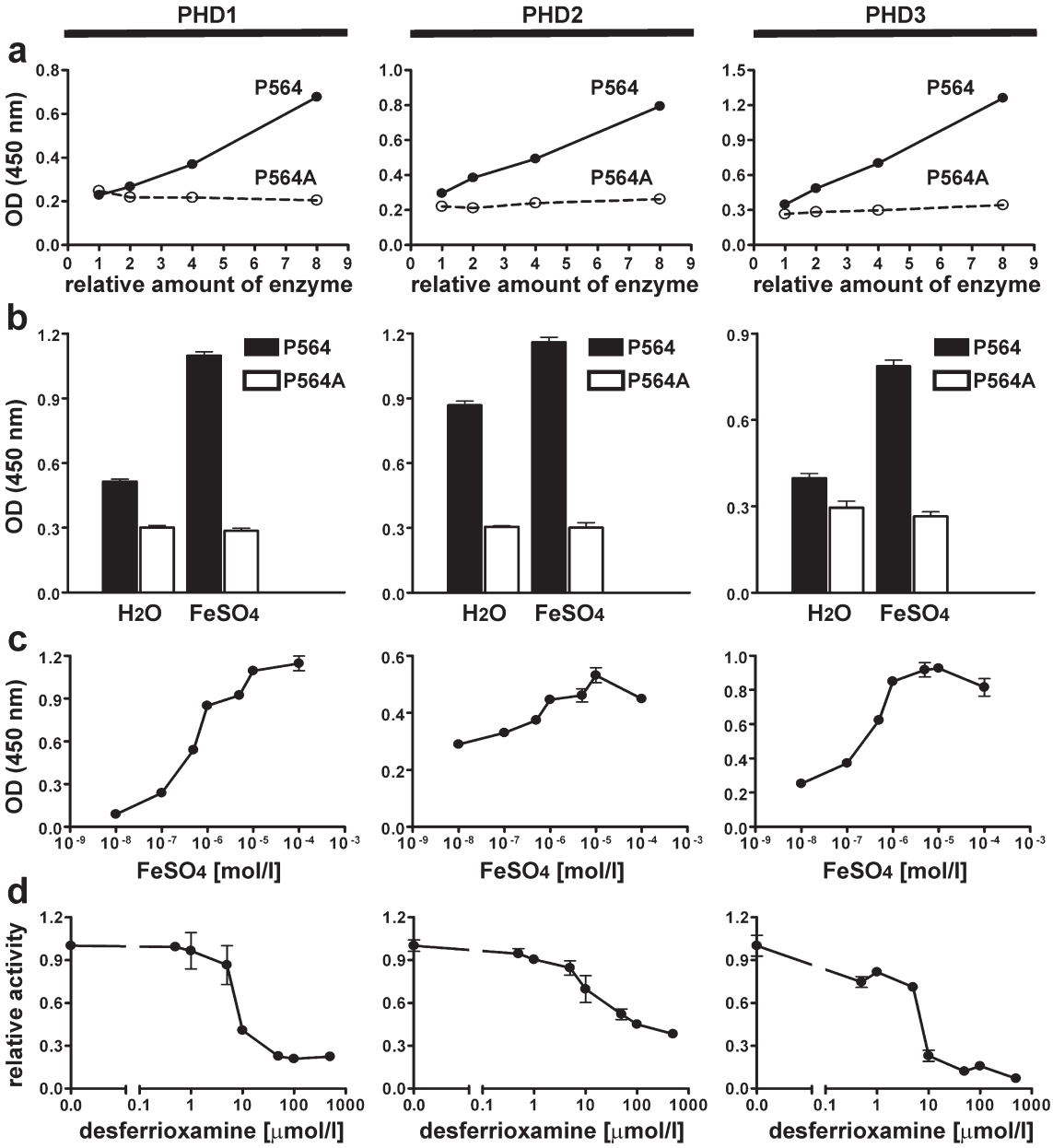

FIG. 1. Regulation of PHD function by iron. PHD1, PHD2, and PHD3 (*from left to right*) were purified as GST-tagged fusion-proteins from Sf9 cells and their hydroxylation activity was estimated in microtiter plate-based VBC binding assays. (**A**) VBC binding is dependent on the presence of both functional PHD enzyme and Pro564. Mutant P564A peptides cannot be hydroxylated. (**B**) Ferrous iron supplementation is essential for full PHD function. (**C**) Titration of ferrous iron reveals that ~10 μM $FeSO_4$ is required for full PHD function. (**D**) Inhibition of PHD function by iron chelation. Mean values ± SEM of representative experiments performed in triplicate are shown.

(Hyp)564 peptide-containing hydroxylation assays and subsequently diluted to obtain solutions with similar specific activities which were then used for all following experiments.

Although 2 μM $FeSO_4$ was initially present in the elution buffer, the PHDs did not display full activity without additional ferrous iron in the reaction buffer (Fig. 1B), suggesting that both active center and added iron was at least partially oxidized. An iron titration experiment revealed that the addition of ~10 μM $FeSO_4$ was required for full induction of the hydroxylation activity of all three PHDs (Fig. 1C). This concentration was hence kept in the following experiments. The addition of ferric iron did not stimulate the activity of the PHDs (data not shown).

The sensitivity of the PHDs to the availability of "free" iron explains the long-known feature of iron chelators to induce the HIFα subunits. Indeed, the hexadentate hydroxamic acid iron chelator desferrioxamine inhibited the *in vitro* hydroxylation activity of all three PHDs (Fig. 1D). Of note, PHD2 was some-

what less sensitive to desferrioxamine than PHD1 or PHD3, which were efficiently inhibited by 10 μM desferrioxamine, corresponding to the concentration of iron in the reaction solution.

Requirement for 2-oxoglutarate and inhibition by succinate

2-Oxoglutarate is used as a co-substrate of all three PHDs which is oxidatively decarboxylated during target protein hydroxylation. Titration experiments with 2-oxoglutarate revealed that all three PHDs required a similar 2-oxoglutarate concentration of ~10–100 μM for full activity (Fig. 2A). These data confirm previous estimates of Km values of 55–60 μM for all three PHDs (17), and a PHD2 binding constant for 2-oxoglutarate of <2 μM (36). Therefore, a concentration of 500 μM 2-oxoglutarate was kept in all further experiments. Higher concentrations of 2-oxoglutarate inhibited the *in vitro* PHD activity in some experiments. Whether this effect is of physiological relevance is currently unknown.

It has been reported previously that succinate can inhibit PHD activity (12, 30, 48). We hence tested the effects of increasing succinate concentration on each PHD enzyme. In the presence of 500 μM 2-oxoglutarate, addition of succinate only moderately inhibited the hydroxylation activity of purified PHDs and this effect was even reversed at higher concentrations (Fig. 2B). However, in the presence of 50 μM 2-oxoglutarate, succinate efficiently inhibited PHD activity with IC_{50} values of ~600 μM (Fig. 2C).

Vitamin C oxidation impairs PHD function

Vitamin C is essential for the function of collagen hydroxylases as well as for HIFα hydroxylases because it protects the enzyme's amino acid residues and/or active center iron from oxidation in reactions uncoupled from target hydroxylation (38). Purified PHDs do not show any activity in the absence of ascorbate and the addition of at least 0.1 mM ascorbate was required to fully induce hydroxylation activity of all three PHDs (Fig. 3A). The addition of oxidized ascorbate (dehydroascorbate) did not induce PHD activity (data not shown). These data confirm previously reported similar ascorbate Km values (140–180 μM) for all three PHDs (17). Therefore, an excess of freshly prepared ascorbate (2 mM) was added to all subsequent

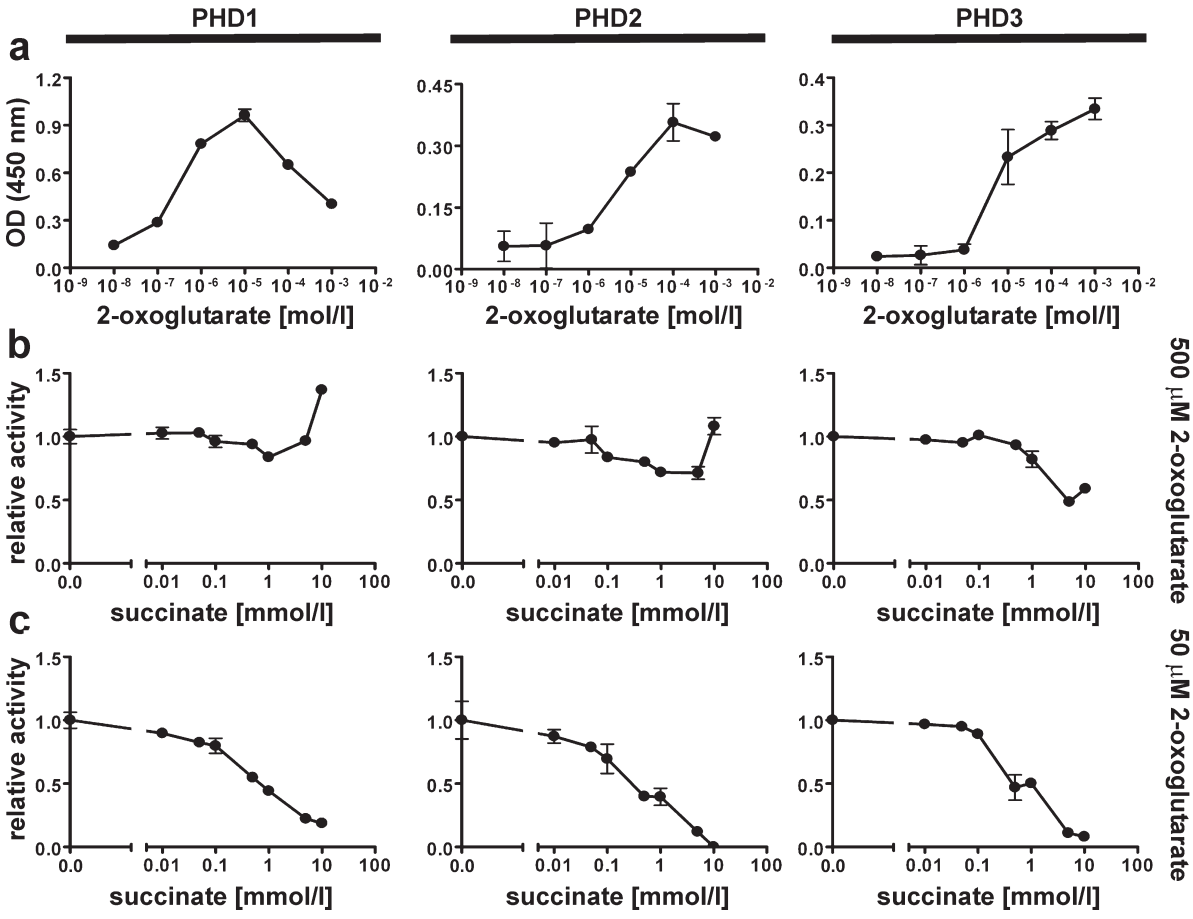

FIG. 2. Regulation of PHD function by Krebs cycle intermediates. PHD1, PHD2, and PHD3 (*from left to right*) hydroxylation activity was estimated in microtiter plate-based VBC binding assays. Titration curves of the cosubstrate 2-oxoglutarate (**A**) or the co-product succinate in the presence of 500 μM (**B**), or 50 μM (**C**) 2-oxoglutarate are shown as mean values ± SEM of representative experiments performed in triplicate.

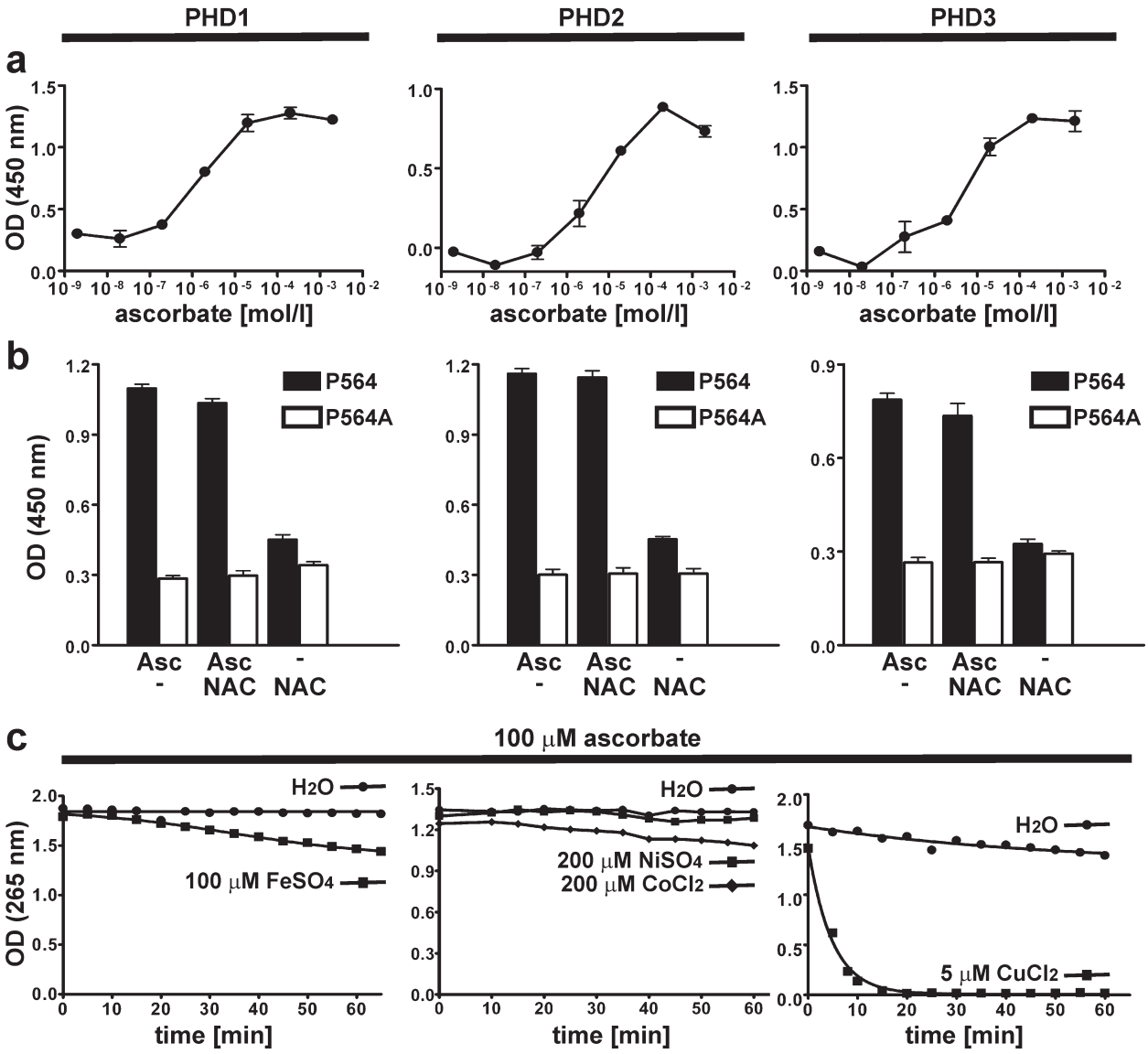

FIG. 3. Regulation of PHD function by vitamin C. PHD1, PHD2, and PHD3 (*from left to right*) hydroxylation activity was estimated in microtiter plate-based VBC binding assays. (**A**) Titration of vitamin C reveals that 0.1 m*M* ascorbate is minimally required for PHD function. (**B**) The antioxidant *N*-acetyl-L-cysteine (250 μM) cannot replace ascorbate (2 m*M*) for PHD function. (**C**) Ascorbate is slowly oxidized by air (dehydroascorbate does not absorb light at 265 nm). Ferrous iron slightly catalyzes this reaction (*left graph*), whereas the effects of the known PHD inhibitors cobalt and nickel are negligible (*middle graph*). However, cupric copper strongly catalyzed ascrobate oxidation (*right graph*). Mean values ± SEM of representative experiments performed in triplicate are shown.

experiments. Antioxidative ascorbate function is specifically required by the PHDs and cannot be replaced by the antioxidant *N*-acetyl-L-cysteine (Fig. 3B).

The absolute requirement for ascorbate might also explain the function of previously reported agents inhibiting PHD activity. Ascorbate oxidation by air can be measured by the drop in absorbance at 265 nm. However, as shown in Fig. 3C, ascorbate oxidation is negligible during the reaction period of 1 h. Even in the presence of equimolar ferrous iron, the ascorbate levels dropped only by 20% after 1 h (Fig. 3C, left graph). The transition metals cobalt and nickel are well-known inducers of HIFα protein stability due to PHD inhibition. Of note, even a twofold molar excess of $CoCl_2$ or $NiSO_4$ did not catalyze more ascorbate oxidation than $FeSO_4$ (Fig. 3C, middle graph). In contrast, copper efficiently catalyzed ascorbate oxidation: 5 μM $CuCl_2$ destroyed >90% of 100 μM ascorbate within 10 min (Fig. 3C, right graph).

The antioxidants gallic acid and n-propyl gallate efficiently inhibit the activity of all three PHDs

A number of natural nutrient compounds has been suggested to induce HIF-1α; among them flavonoid and nonflavonoid polyphenols. Catechins are nonflavonoid polyphenols found in

green tea leaves which have been reported to induce HIF-1α (60). Only catechins containing a 3-gallate moiety activate HIF-1 and have been shown to inhibit PHD2 activity (52, 53). Thus, we tested the sensitivity of PHD-dependent peptide hydroxylation on gallic acid or *n*-propyl gallate. Remarkably, all three PHDs were efficiently inhibited by 33–100 μM gallic acid (Fig. 4A) or by 3.3–10 μM *n*-propyl gallate (Fig. 4B). As shown in Fig. 4C, n-propyl gallate also induced HIF-dependent luciferase reporter gene activity in cultured CHO cells. In contrast to the *in vitro* results, gallic acid treatment of cells only marginally affected luciferase expression (data not shown).

DISCUSSION

Oxygen sensing by protein hydroxylation is a regulated process

Sensing cellular pO_2 is probably not the only function of the PHDs. Otherwise, an excess of a single sensory protein simply regulated by oxygen availability would be all that was required for this task. However, there is ample evidence that the PHD oxygen sensors fulfill additional functions. First, different tissues are differently vascularized, unequally perfused, and show spatially and temporally variable oxygen consumption rates. Thus, the mean pO_2 varies from tissue to tissue, if not from cell to cell. Nevertheless, every cell is capable of sensing a reduction in oxygenation and adequately responds to such a reduction by inducing HIF-dependent gene expression. Therefore, also hypoxia thresholds vary both spatially and temporally, and the PHD oxygen sensors evolved to meet these requirements by showing a variable tissue-specific expression pattern. Second, every successful adaptation to hypoxia eventually results in reoxygenation of the affected tissue. Because following hypoxia there are much higher levels of HIFα in the cell, the oxygen-dependent hydroxylation and degradation machinery must increase its capacity to cope with the degradation of the increased protein mass. Third, HIFα is also induced by a variety of stimuli under normoxic conditions. How can HIFα protein stabilization occur in the presence of active PHDs and sufficient oxygen supply? A plausible explanation lies in the lim-

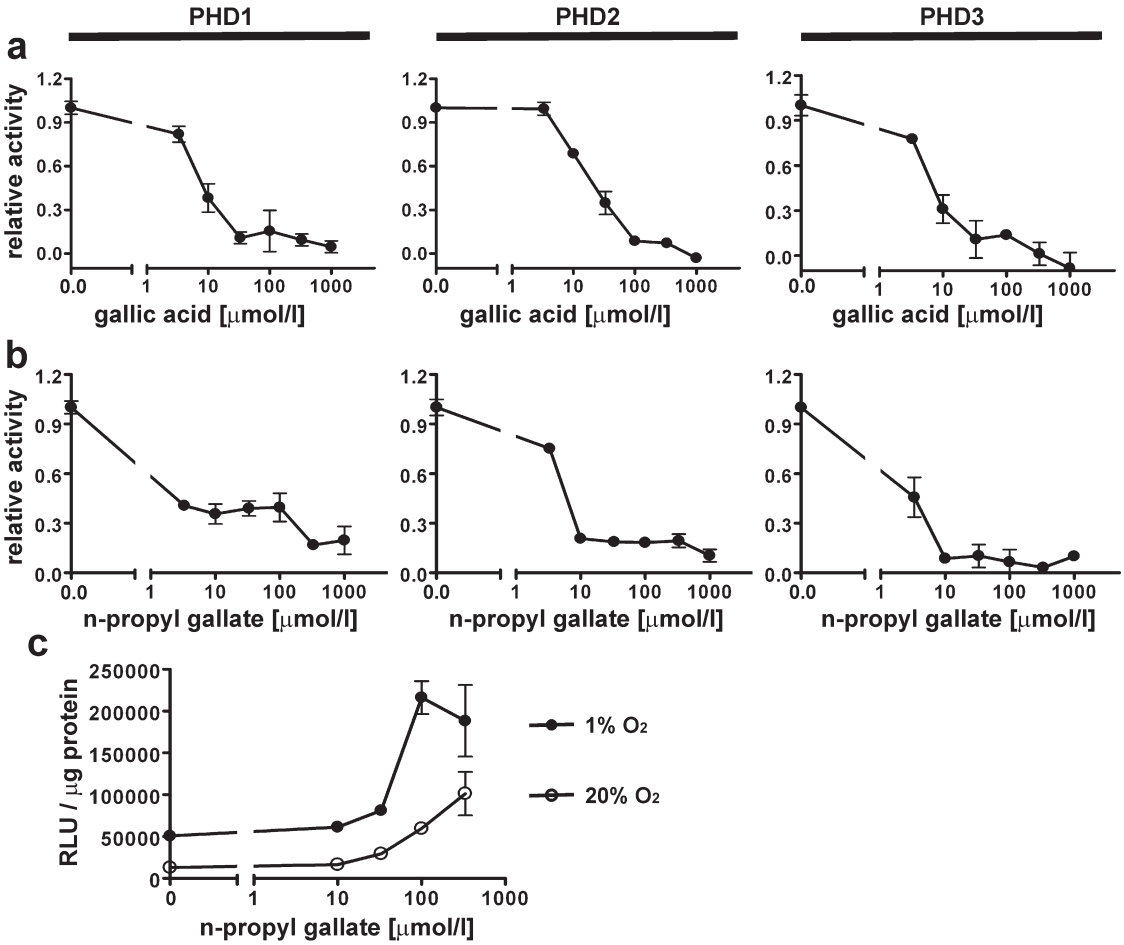

FIG. 4. Regulation of PHD function by gallate. The effects of gallic acid (**A**) and *n*-propyl gallate (**B**) on PHD1, PHD2, and PHD3 (*from left to right*) hydroxylation activity were estimated in microtiter plate-based VBC binding assays. (**C**) CHO cells stably transfected with a HIF-dependent luciferase reporter gene were treated with the indicated concentrations of *n*-propyl gallate for 24 h. Relative light units (RLU) were normalized to the protein content of the lysates. Mean values ± SEM of representative experiments performed in triplicate (quadruplicates for the reporter gene assays) are shown.

ited expression levels of PHDs which are finely counterbalanced by HIFα levels. If either one is upregulated, it overcomes the function of the other. Thus, as well as an increase in PHD synthesis leads to HIFα degradation, an increase in HIFα synthesis leads to its stabilization even under normoxic conditions. We have previously demonstrated that these mechanisms work even under hypoxic conditions, since PHD-dependent HIFα hydroxylation was reduced but still functional in direct proportion to the decrease in available oxygen (50).

Regulation of the three PHD oxygen sensors by small molecules

In this work, we have analyzed the effects of several small molecules on PHD function *in vitro*. Addition of ferrous iron was essential for PHD activity. Hirsilä *et al.* reported iron Km values for PHD1, PHD2, and PHD3 of 30, 30, and 100 n*M*, respectively (18), and McNeill *et al.* reported a ferrous iron binding constant of highly purified PHD2 of $<<1$ μM (36). Our data support an increased iron affinity of PHD3 that reached maximal activity already in the presence of 1 μM $FeSO_4$, whereas PHD1 and PHD2 required 10 μM $FeSO_4$ for full activity. The iron chelator desferrioxamine efficiently inhibited PHD1 and PHD3 activity at concentrations corresponding to the iron concentrations in the reaction buffer. PHD2 inhibition was somewhat less efficient, but a drop in activity was observed with similar desferrioxamine concentrations. Interestingly, it has previously been suggested that desferrioxamine inefficiently inhibited crude PHD enzymes *in vitro*: in the presence of 5 μM Fe^{2+} up to 1 m*M* desferrioxamine inhibited crude PHDs by $<20\%$, whereas inhibition of pure PHDs was much more efficient (18). Thus, the efficiency of inhibition of the PHDs by desferrioxamine might allow us to draw some conclusions on the purity of the enzyme preparation.

We previously determined an iron concentration in the FCS of 148 μM and in the cell culture medium of 16 μM (56). The intracellular concentration of chelatable iron has been reported to be 3–6 μM, depending on the cell type (45). Thus, the estimated concentration of "free" iron for optimal PHD activity *in vitro* corresponds to the iron concentrations *in vivo*, suggesting that iron availability is indeed able to regulate the HIF system by influencing PHD activity. This assumption has been confirmed by experimentally changing the "labile iron pool" in cancer cells (22, 24, 25). Interestingly, important iron uptake and transport proteins are among the targets of HIF-1 that might function also as a regulator of iron homeostasis in addition to oxygen homeostasis (58). These values also explain why ~100 μM of the hexadentate extracellular iron chelator desferrioxamine but only ~10 μM of the bidentate intracellular iron chelator cyclopirox olamine were required to induce the HIF system by inhibiting PHD activity (29).

Krebs cycle intermediates such as succinate and fumarate are known to induce the HIF system by inhibiting PHD activity (20, 43, 48). Moreover, 2-oxoacids such as pyruvate and oxaloacetate also induce HIFα (12, 30). These findings link mitochondrial function and cellular metabolism with the PHD/HIF oxygen sensing system. Mutation of the tumor suppressor *succinate dehydrogenase* has been reported to increase cellular succinate concentration from 120 to ~440 μM (48). The IC_{50} values for succinate have been reported to be in the range of 510–830 μM for purified PHDs (26). We obtained similar IC_{50} values (~600 μM) for succinate when the assays were performed in the presence of 50 μM 2-oxoglutarate. Succinate competes for 2-oxoglutarate binding to the PHDs (16, 26), explaining why we observed only weak succinate inhibition of PHD activity in the presence of high (500 μM) 2-oxoglutarate concentrations.

We demonstrated that ascorbate is essential for the function of all three PHDs. It has been shown that physiological concentrations (25 μM) of ascorbate suppress HIF-1α protein levels in cancer cells, suggesting that intracellular ascorbate concentrations indeed respresent a major regulator of PHD function (25). Ascorbate depeletion thus could also explain how transition metals induce HIF-1α. Interestingly, Salnikow and colleagues reported that cellular ascorbate depletion causes $NiSO_4$ and $CoCl_2$ induced HIF-1α stabilization (23, 46). However, the authors attributed this effect to inhibition of cellular ascorbate uptake. Moreover, we could not observe any relevant nickel- or cobalt-mediated degradation of ascorbate *in vitro*. In contrast, we observed a rapid copper-mediated ascorbate oxidation which most likely explains the previously reported $CuCl_2$-induced HIF-1α stabilization via PHD inhibition in cultured cells (32).

We have found that the antioxidant compounds gallic acid and *n*-propyl gallate efficiently inhibited all three PHDs *in vitro* and the esterified gallate also induced a HIF-dependent reporter gene in cell culture. Tsukiyama *et al.* hypothesized that two

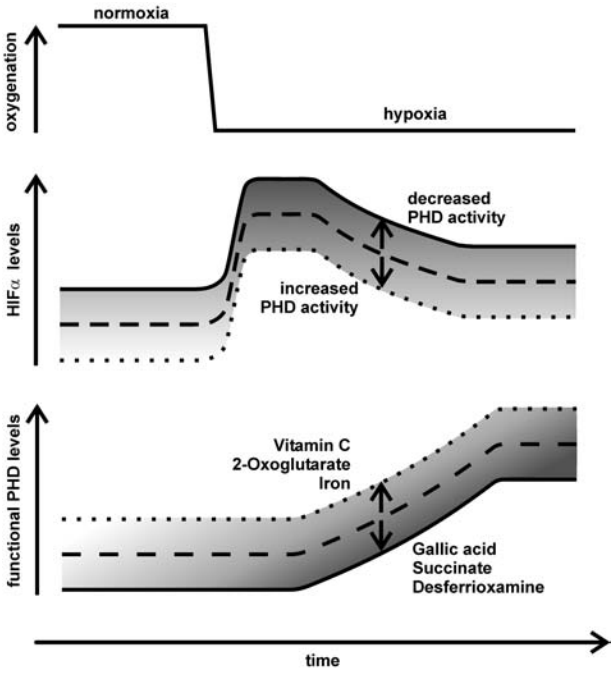

FIG. 5. Regulation of PHD-dependent oxygen sensing. Upon a hypoxic insult, the HIF-dependent transcriptional increase of PHD2 and PHD3 levels provides a negative feedback mechanism that defines a novel, adapted oxygen sensing setpoint capable of reacting to a second hypoxic insult. Small molecules contribute to the regulated oxygen sensing by triggering PHD function, leading to an inverse regulation of the HIF levels (for details see text).

phenolate oxygen atoms of gallate chelate with the active center iron and the carboxyl group of gallate forms a strong ionic/hydrogen bonding with Arg383 of PHD2 (53). In the hands of these authors, *n*-propyl gallate was effective only in cell culture but not in cell-free PHD assays, which was attributed to the esterified carboxyl group of *n*-propyl gallate. However, in our hands *n*-propyl gallate was even more efficient in PHD inhibition *in vitro* than gallic acid. A likely reason for this discrepancy was the use of crude cell lysates in a 2-oxoglutarate conversion assay by these authors. We recently found that 2-oxoglutarate turnover using crude lysates is independent of PHD function (Wirthner R. *et al.*, unpublished observations).

What might be the mechanism of PHD inhibition by gallate-containing antioxidants in comparison to other antioxidants such as *N*-acetyl-L-cysteine? The use of molecular dioxygen for oxidative decarboxylation-coupled protein hydroxylation requires short-lived, highly reactive transition states of oxygen. Antioxidants fitting into the active center of PHDs might scavenge these ROS, thereby blocking the PHD reaction cycle. Similarly, exogenously produced ROS potentially could interfere with these reactive transition states of oxygen. It has been suggested that the increase in ROS content in $junD^{-/-}$ or mucin 1 (MUC1) knock-down cells leads to a decrease in PHD activity and hence to HIF-1α accumulation (15, 59). Thus, antioxidants paradoxically would have the potential to do both: protect PHD function from exogenous ROS and block PHD activity by destruction of PHD-inherent ROS intermediates. The effective function of a given antioxidant would depend on the accessibility to the active center, interference with transition metals and ascorbate, and the scavenging efficiency of mitochondrial and/or NADPH oxidase-derived ROS.

Regulation of the PHD oxygen sensors by other mechanisms

Up to date, only few reports deal with the regulation of PHDs by protein–protein interactions. The E3 ubiquitin ligase Siah2 regulates PHD1 and PHD3, but not PHD2, protein stability (39). PHD3, but not PHD1 or PHD2, appears to be a substrate for the TRiC chaperonin (34). OS-9 apparently is simultaneously interacting with both HIFα and PHD2 or PHD3, but not PHD1, thereby enhancing HIFα hydroxylation and degradation (4). Mitogen-activated protein kinase organizer 1 (MORG 1) might provide the molecular scaffold for HIFα interaction specifically with PHD3 (19). Finally, we recently reported that FKBP38 specifically regulates the stability of PHD2 (5). These examples demonstrate two things: first, abundance and function of PHDs can also be regulated by specific proteins; and second, the three different PHDs are regulated in nonidentical ways, further supporting their nonredundant role in oxygen sensing. While the cell-free *in vitro* assay used in this work provides useful information on the regulated function of each PHD individually, it can of course not take into account the manifold additional modes of PHD regulation, (*e.g.*, by protein–protein interaction), found within a cell.

PHD2 and PHD3, but not PHD1 or FIH, are transcriptionally induced under hypoxic conditions (3, 6, 8, 11, 13, 14, 33). It could be shown that HIF is required for hypoxic induction of PHD2 and PHD3 gene expression, and hypoxia response elements were identified in the regulatory regions of the corresponding genes (37, 42). Because the essential cofactor oxygen is basically lacking under hypoxic conditions, the HIF-dependent hypoxic increase in PHD abundance is somewhat paradoxical. It has been suggested that increased PHD levels accelerate the termination of the HIF response following reoxygenation (2, 3, 14, 33). Indeed, biochemical *in vitro* studies revealed K_m values of purified PHDs for oxygen close to the pO_2 in air, suggesting that the kinetics of specific HIFα hydroxylation under hypoxic conditions are rather slow (17). However, tissues *in situ* have to deal with a great variability of generally very low pO_2 values, even when the inspiratory pO_2 is considered to be "normoxic". Thus, the PHD oxygen sensors need to operate at different pO_2 setpoint values in different tissues. We recently demonstrated that a self-regulatory loop defines a specific threshold for HIFα-activation as a function of the actual pO_2 (50). As schematically outlined in Fig. 5, this negative feedback-loop includes: (a) a HIF-dependent induction of PHD2 and PHD3 upon a reduction in oxygen supply; (b) a PHD-dependent, partial reduction of HIFα even under very low pO_2; (c) the subsequent partial reduction of the PHD2 and PHD3 levels; (d) the definition of a novel setpoint for oxygen sensing by leveling off the HIF/PHD ratio; (e) a secondary response to a further, more severe hypoxic insult; and (f) the triggering by iron, ascorbate, Krebs cycle intermediates, ROS, and antioxidants. Small molecule regulators of PHD activity are thus functionally relevant under normoxic as well as hypoxic conditions.

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ABBREVIATIONS

FIH, factor inhibiting HIF; HIF, hypoxia-inducible factor; Hyp, hydroxyproline; ODD, oxygen-dependent degradation; PHD, prolyl-4-hydroxylase domain protein; pO_2, oxygen partial pressure; pVHL, von Hippel–Lindau tumor suppressor protein; ROS, reactive oxygen species; VBC, pVHL/elongin B/elongin C.

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Vitamin C is dispensable for oxygen sensing in vivo

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Prolyl-4-hydroxylation is necessary for proper structural assembly of collagens and oxygen-dependent protein stability of hypoxia-inducible transcription factors (HIFs). In vitro function of HIF prolyl-4-hydroxylase domain (PHD) enzymes requires oxygen and 2-oxoglutarate as cosubstrates with iron(II) and vitamin C serving as cofactors. Although vitamin C deficiency is known to cause the collagen-disassembly disease scurvy, it is unclear whether cellular oxygen sensing is similarly affected. Here, we report that vitamin C–deprived *Gulo*$^{-/-}$ knockout mice show normal HIF-dependent gene expression. The systemic response of *Gulo*$^{-/-}$ animals to inspiratory hypoxia, as measured by plasma erythropoietin levels, was similar to that of animals supplemented with vitamin C. Hypoxic HIF induction was also essentially normal under serum- and vitamin C–free cell-culture conditions, suggesting that vitamin C is not required for oxygen sensing in vivo. Glutathione was found to fully substitute for vitamin C requirement of all 3 PHD isoforms in vitro. Consistently, glutathione also reduced HIF-1α protein levels, transactivation activity, and endogenous target gene expression in cells exposed to $CoCl_2$. A Cys201Ser mutation in PHD2 increased basal hydroxylation rates and conferred resistance to oxidative damage in vitro, suggesting that this surface-accessible PHD2 cysteine residue is a target of antioxidative protection by vitamin C and glutathione. (*Blood*. 2011;117(20): 5485-5493)

Introduction

Oxygen is essential for several physiologic processes, particularly for cellular respiration and energy metabolism. On the molecular level, response to hypoxia is mediated by hypoxia-inducible transcription factors (HIFs). Under continuous oxygen supply, 2 distinct prolyl residues within the oxygen-dependent degradation domain of HIFα subunits are hydroxylated by prolyl-4-hydroxylase domain–containing enzymes (PHDs). Hydroxy-HIFα is recognized by the von Hippel–Lindau tumor suppressor protein (pVHL) and subsequently targeted for proteasomal degradation.[1,2] When oxygen is limited, PHD activity ceases, and nonhydroxylated HIFα is stabilized and heterodimerizes with the HIFβ subunit to activate expression of numerous target genes.[3] Moreover, an asparaginyl hydroxylase termed factor-inhibiting HIF hydroxylates a C-terminal Asn residue of HIFα subunits in an oxygen-dependent manner, thereby regulating cofactor recruitment and HIFs' transcriptional activity.[4]

Three PHD isoforms have been characterized so far, termed PHD1, PHD2, and PHD3; they differ in size, subcellular localization, and tissue distribution.[5] PHD2 is the most ubiquitously expressed isoform, responsible for the normoxic control of HIFα.[6] Accordingly, genetic ablation of PHD2 but not PHD1 or PHD3 results in embryonic lethality in mice.[7] Suggesting a fundamental role in the hematopoietic and circulatory systems, somatic inactivation of PHD2 leads to increased erythropoiesis and angiogenesis as a result of HIFα stabilization followed by activation of its target genes, including erythropoietin (EPO) and vascular endothelial growth factor.[8] Knockout of either PHD1 or PHD3 had no effect on hematologic parameters. However, combined PHD1/PHD3 knockout animals showed a slight increase in hematocrit, hemoglobin, and red blood cell counts.[9] Clinical data on patients with erythrocytosis revealed Pro317Arg or Arg371His mutations in the gene encoding for PHD2, altering the hydroxylation efficiency of the mutant protein.[10,11] A third point mutation in PHD2 (His374Arg) was found in a patient suffering from erythrocytosis and paraganglioma.[12] These case reports emphasize the critical role of PHD2 in regulating erythropoiesis and maintaining red blood cell homeostasis in humans.

PHDs belong to a larger superfamily of 2-oxoglutarate and Fe(II)-dependent dioxygenases. Similar to collagen prolyl-4-hydroxylase ([C-P4H]; EC 1.14.11.2), PHDs require molecular oxygen and 2-oxoglutarate as cosubstrates, as well as ferrous iron and probably L-ascorbic acid (vitamin C) as cofactors for enzymatic activity.[13] K_m values of PHDs for oxygen are strikingly higher than those of other prolyl-4-hydroxylases.[13] The relatively low oxygen affinity is essential for effective oxygen sensing, because even small changes in oxygen partial pressure can influence hydroxylation activity.[13-15]

In a previous study, we reported on the dose-dependent regulation of the in vitro activity of all 3 PHD isoforms by their essential cosubstrates and cofactors, including vitamin C.[16] Primates, including humans, lost the ability to synthesize vitamin C de novo and thus depend on dietary vitamin C intake. C-P4Hs hydroxylate proline residues to stabilize the collagen triple helix structure. Because ascorbate is an essential cofactor for C-P4Hs, persistent ascorbate deficiency results in disassembly of connective tissue structures, a common symptom of the nowadays rare disease scurvy.[17] With K_m values ranging from 140 to 180μM, the

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requirement of PHDs for vitamin C in vitro is only 2-fold lower than for C-P4H, suggesting that HIF hydroxylases also could well be affected by vitamin C malnutrition.[13] Mice lacking a functional *Gulo* gene have been described as a model to study vitamin C deficiency.[18] *Gulo* encodes for l-gulono-1,4-lactone-oxidase (EC 1.1.3.8), a key enzyme involved in the final step of vitamin C biosynthesis. Dietary vitamin C deprivation leads to body weight loss, anemia, aortic wall damage, and internal hemorrhages in these mice.[18]

Although the interaction between the target prolyl residue, molecular oxygen, 2-oxoglutarate, and iron during the reaction cycle in the active center of PHDs has been described in detail previously, the apparently inevitable presence of vitamin C for the in vitro function of the PHDs remains elusive.[19,20] Because of its antioxidative properties, vitamin C might maintain ferrous iron in the reduced state. Given the enzymatic relationship between HIFα and C-P4Hs, we set out to investigate the effect of dietary vitamin C on the regulation of the PHD-HIF oxygen–sensing pathway in $Gulo^{-/-}$ mice under normoxemic and hypoxemic conditions.

Methods

Cell culture

HeLa human cervix carcinoma cells were adapted to Ham nutrient mixture F-12 (Sigma-Aldrich), free of ascorbate and fetal calf serum (FCS), containing the following supplements: epidermal growth factor (50 ng/mL; Sigma-Aldrich), insulin (5 μg/mL; Sigma-Aldrich), apo-transferrin (5 μg/mL; Sigma-Aldrich), hydrocortisone (100nM; Sigma-Aldrich) buffered with 15mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid pH 7.4 as well as 100 IU/mL penicillin and 100 μg/mL streptomycin. HepG2 human hepatoma cells stably transfected with a HIF-dependent firefly luciferase reporter gene termed HRG1 have been described previously.[21] If not indicated otherwise, all cells were maintained in DMEM with 10% FCS, 100 IU/mL penicillin, and 100 μg/mL streptomycin. Cell number and viability were determined using a ViCell counter (Beckman Coulter).

HIF transactivation activity

HeLa cells (5×10^5) were cotransfected with 500 ng of the HIF-dependent pH3SVL reporter vector containing 6 HIF binding sites in total derived from regulatory elements of the *transferrin* gene[22] and 40 ng of pRL-CMV *Renilla* luciferase expression plasmid (Promega) essentially as described previously.[14] Twenty-four hours after transfection, cells were split and exposed to graded oxygen concentrations (21%-0.2% oxygen) for 24 hours by using cross-calibrated oxygen-controlled CO_2 incubators (CB 150; Binder). Stably transfected HRG1 HIF reporter cells were adapted to 1% FCS overnight and treated with 50μM desferrioxamine mesylate (Dfx; Sigma-Aldrich) or 100μM $CoCl_2$ and 1 to 10mM reduced l-γ-glutamyl-l-cysteinyl-glycine ([GSH] 250mM stock solution adjusted to pH 7.0) or 0.2 to 2mM ascorbate for 24 hours. For hypoxic experiments, cells were grown under 2% O_2 for 24 hours and treated with GSH or ascorbate. HRG1 cells were transfected with pRL-SV40 *Renilla* luciferase to control for non–HIF-mediated effects of ascorbate and GSH on the heterologous simian virus 40 minimal promoter present in both constructs. Cells were lysed using passive lysis buffer, and luciferase activities were determined according to the manufacturer's instructions (Promega) by using a 96-well luminometer (Berthold Technologies). Data are expressed as relative luciferase activities per total cellular protein of experiments performed in triplicates by calculating the ratio of firefly/*Renilla* activities per well.

Expression and purification of recombinant PHD enzymes

Recombinant PHD proteins were expressed and purified as glutathione transferase (GST)–fusion proteins from baculovirus-infected Sf9 insect cells as described previously.[14] Untagged enzyme preparations were obtained by introducing a PreScission protease cleavage site between the GST-tag and the PHD open reading frame. A Cys201Ser point mutation was introduced into the human PHD2 expression plasmid by site-directed mutagenesis (Stratagene). Untagged PHD2 was expressed in Sf9 cells and purified by conventional ion-exchange chromatography (kind gift of Dr Felix Oehme, Bayer Healthcare, Wuppertal, Germany). Purity of the enzyme preparations was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by Coomassie staining or immunoblotting.

Prolyl-4-hydroxylation assay

Activity of recombinant PHD enzymes was measured by a microtiter plate-based peptide hydroxylation assay as described previously.[23] In brief, recombinant PHDs were used to hydroxylate a biotinylated peptide derived from HIF-1α (amino acid residues 556-574) coupled to streptavidin-coated 96-well plates. Hydroxylation reaction was performed for 1 hour at room temperature in the presence of 10μM $FeSO_4$, 0.5mM 2-oxoglutarate, and 2mM ascorbate in 20mM Tris-HCl pH 7.5, 5mM KCl, and 1.5mM $MgCl_2$. Hydroxylated peptides were detected by recombinant, thioredoxin-tagged von Hippel–Lindau/elongin B/elongin C (VBC) complex. Reactions were stopped by removing the reaction mix and adding 1mM H_2O_2. Bound VBC complex was detected by rabbit anti-thioredoxin antibodies and secondary horseradish peroxidase (HRP)–conjugated anti-rabbit antibodies (Sigma-Aldrich) by using the 3,3,5,5-tetramethylbenzidine substrate kit (Pierce). The peroxidase reaction was stopped by adding 2M H_2SO_4, and absorbance was determined at 450 nm in a microplate reader. Background values as determined by using a mutant HIF-1α (Pro564Ala) peptide were subtracted for each experiment.

Ascorbate determination

Ascorbate content of in vitro hydroxylation assay samples was quantified by high-performance liquid chromatography (HPLC) as described previously.[24] In brief, a 10-fold dilution of the enzyme reaction mix containing 2mM ascorbate was analyzed before and after 1 hour of hydroxylation reaction. After dilution in the mobile phase (60mM phosphoric acid, pH 3.1), a 20-μL sample was injected onto a Nucleosil C18 column (Macherey Nagel) and eluted applying an acetonitrile gradient (0%-60%). Ascorbate elution was monitored at 254 nm, corresponding to 96% absorbance of ascorbate and only 4% of dehydroascorbate.[25] Chromatograms and standard curve of pure ascorbate ranging from 25 to 200μM were used to calculate the content of ascorbate in study samples (supplemental Figure 2, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Ascorbate levels in plasma samples of mice were determined by HPLC (Swiss Vitamin Institute).

Immunoblot analyses

Total soluble cellular proteins were extracted with a high salt extraction buffer containing 0.4M NaCl, 0.1% Nonidet P-40, 10mM Tris-HCl pH 8.0, 1mM ethylenediaminetetraacetic acid, 1mM dithiothreitol, 1mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (Sigma-Aldrich). Protein concentration was measured by the method of Bradford, and 60 to 70 μg of cellular protein was subjected to immunoblot analyses. Membranes were probed using the following dilutions of mouse monoclonal (mAb) or rabbit polyclonal antibodies: mAb anti–HIF-1α (1:1000; BD Transduction Laboratories), mAb anti-CA9 (M75; kindly provided by S. Pastorekova, Bratislava, Slovak Republic), mAb anti–β-actin (1:5000; Sigma-Aldrich), rabbit anti-PHD1 (1:2000; Genway), rabbit anti-PHD2 (1:1000; Novus), or rabbit anti-PHD3 (1:1000; Novus), followed by secondary HRP-conjugated antibodies (1:2000; all Pierce).

mRNA quantification

Total RNA purification and mRNA determination by real-time PCR has been described previously.[14] Transcript levels of the HIF-dependent and -independent genes were quantified by reverse transcription (RT) quantitative (q) PCR using SYBR Green qPCR reagent kit (Sigma-Aldrich) in combination with an MX3000P light cycler (Stratagene). Initial copy number of each sample was calculated by comparison with serial dilutions

of a calibrated standard. For mouse tissues, ribosomal protein S12 mRNA was used as a housekeeping gene, and ribosomal protein L28 mRNA served as control for samples from human cell lines. Primer sequences are given in supplemental Table 1.

OxyBlot detection of protein oxidation

PHD2 carbonylation was determined with a protein oxidation detection kit (OxyBlot; Millipore). In brief, 50 ng/μL recombinant PHD2 was exposed to either 100μM $CoCl_2$, 10μM $FeSO_4$, 2mM ascorbate, 0.5mM 2-oxoglutarate, or 1mM H_2O_2 in the presence of 400nM wild-type or Pro564Ala mutant HIF-1α peptide in 20mM Tris–HCl, 5mM KCl, and 1.5mM $MgCl_2$ for 1 hour at room temperature. Then, 5 μL of the reaction mix was mixed with 5 μL of 12% sodium dodecyl sulfate, and the carbonyl groups were derivatized with 10mM 2,4-dinitrophenylhydrazine for 15 minutes. Dinitrophenyl groups were detected by immunoblotting using rabbit anti-DNP antibodies (1:150) followed by secondary goat anti–rabbit HRP-conjugated antibodies (1:300). For loading controls, PHD2 was detected using rabbit anti-PHD2 antibodies (Novus).

Animal studies

Gulo$^{-/-}$ mice were maintained on vitamin C–supplemented water containing 0.33 g/L L-ascorbic acid and 0.01mM EDTA as described previously.[18] At 3 months of age, ascorbic acid supplement was withdrawn from 6 *Gulo*$^{-/-}$ males but continued for the control males. After 5 weeks, mice were killed with an overdose of 2,2,2-tribromoethanol, and tissues were collected and stored in RNAlater (Applied Biosystems) at −20°C until use. For hypoxia studies, 22 *Gulo*$^{-/-}$ males with an average age of 16 weeks were allocated to 4 groups so that no significant differences were observed in mean body weight and age of the animals at the beginning of the experiment. The body weight was determined every second day. After 36 days of ascorbate withdrawal, mice were exposed to 8% oxygen for 24 hours in a hypoxia tent (Coy Laboratory Products). Control animals were maintained at ambient oxygen concentration. Heparinized whole blood was collected from all mice by cardiac puncture after intraperitoneal injection anesthesia using 4 mg/mL xylazine and 20 mg/mL ketamine at a dosage of 0.1 mL/20 g body weight. Blood samples of hypoxic animals were collected inside the hypoxic tent. Animal experiments were conducted at 2 centers with the appropriate consent by the Institutional Animal Care and Use Committees of the University of North Carolina at Chapel Hill for breeding and normoxic gene expression studies or by the Veterinary Office of the Canton Zürich (119/2010) for hypoxia studies.

Blood parameters and plasma EPO concentrations

Plasma EPO levels were measured by enzyme-linked immunosorbent assay following the procedures recommended by the manufacturer (Quantikine; R&D Systems). EPO concentrations were determined by comparison with a calibrated recombinant mouse EPO standard. Hematologic parameters of mouse whole blood were analyzed by the Division of Hematology (University Hospital, Zürich, Switzerland).

Results

Ascorbate is not required for HIF induction by hypoxia in HeLa cells

HeLa cells have been described previously to grow in serum-free medium supplemented with hormones and growth factors.[26] To achieve cell culture conditions avoiding ascorbate contamination derived from animal sera, HeLa cells were adapted to a chemically defined medium free of ascorbate for at least 2 weeks. Control cells were grown in the same medium supplemented with 50μM ascorbate. Both cell groups proliferated at the same rate with no differences noticed between ascorbate-free and supplemented cells (Figure 1A). Despite its need for PHD activity in vitro, hypoxic

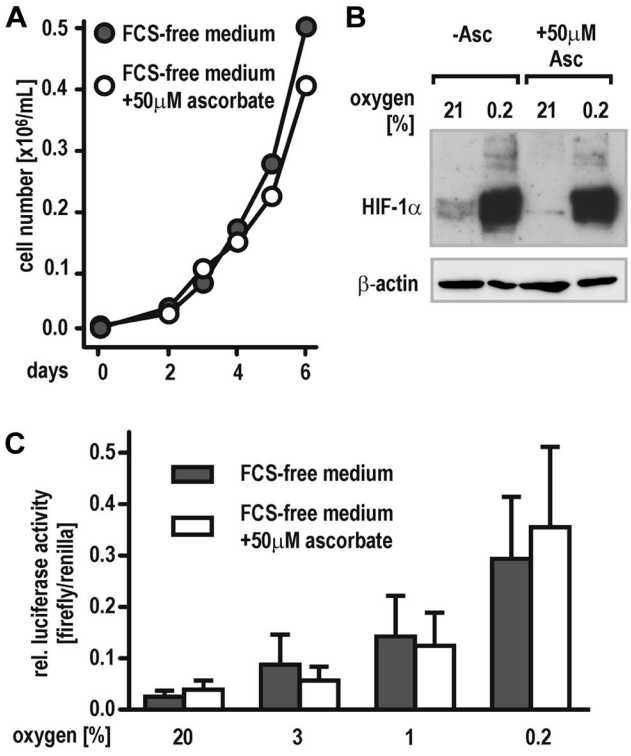

Figure 1. Cellular oxygen sensing by the PHD-HIF pathway does not require vitamin C. (A) Proliferation of HeLa cells growing in FCS-free, chemically defined medium containing either no or 50μM ascorbate. (B) Stabilization of HIF-1α protein in HeLa cells maintained in FCS-free medium containing either no (−Asc.) or 50μM ascorbate. Cells were exposed to 21% and 0.2% oxygen for 6 hours, and then protein levels were analyzed by immunoblotting. (C) Induction of HIF-dependent luciferase activity (pH3SVL vector) in HeLa cells maintained in FCS-free medium containing either no or 50μM ascorbate and exposed to 0.2% to 21% oxygen for 24 hours.

HIF-1α protein accumulation was similar in ascorbate-containing and -deficient cells. However, a faint normoxic induction of HIF-1α could be observed in ascorbate-free cells only (Figure 1B). Accordingly, cells transfected with a HIF-responsive reporter gene (pH3SVL) and subsequently exposed to graded oxygen concentrations (0.2%, 1%, 3%, or 21% O_2, respectively) revealed similar induction levels of luciferase activity under both culture conditions (Figure 1C).

GSH can substitute for vitamin C in the hydroxylation reaction catalyzed by PHDs

Because HeLa cells grown in a medium containing no ascorbate maintained hypoxic HIF-1α stabilization, we speculated that other antioxidants could compensate for vitamin C loss in these cells. Thus, several compounds with antioxidative properties were tested for their effects on PHD hydroxylation activity by using a previously described in vitro hydroxylation assay.[23] Surprisingly, some compounds such as *n*-propyl gallate[16] and the superoxide dismutase mimetic Mn(III) tetrakis(1-methyl-4-pyridyl)porphyrin pentachloride were potent inhibitors of PHD enzymes (supplemental Figure 1A). However, GSH enhanced HIFα hydroxylation by all 3 PHDs. Because the recombinant enzyme preparations in the initial experiments were expressed and purified as GST-fusion proteins, we could not exclude interference of the tested antioxidants, particularly GSH, with the GST-tag. Therefore, a PreScission protease cleavage site was engineered in between the 2 fusion partners. Chimeric GST.PHD and tag-free PHD enzymes showed comparable hydroxylation activity (supplemental Figure 1B). GSH

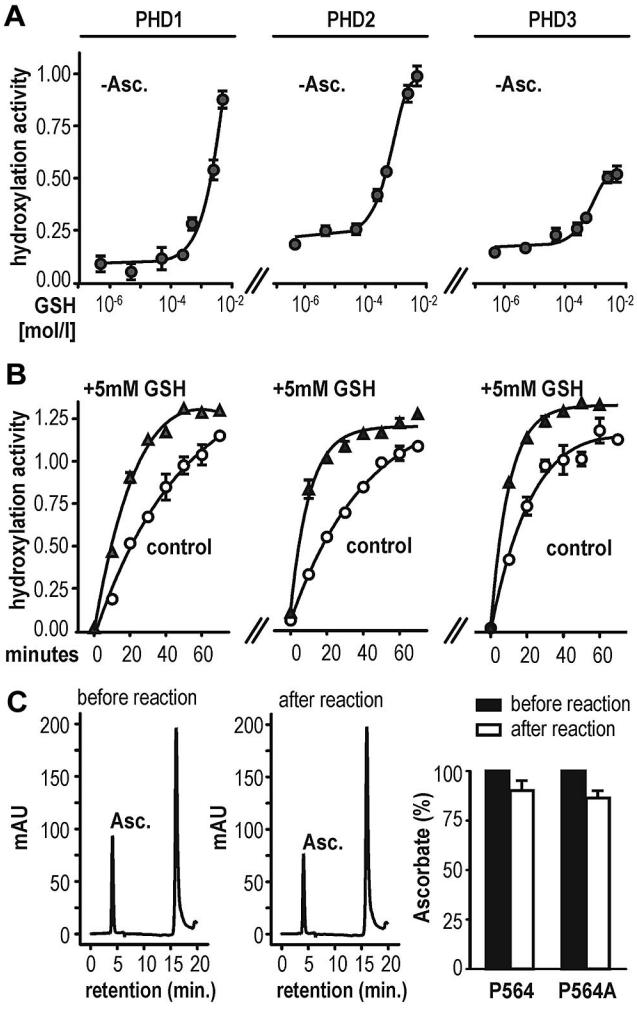

Figure 2. GSH substitutes for vitamin C as a cofactor in HIF-1α hydroxylation in vitro. (A) GSH can enhance PHD hydroxylation activity in the absence of ascorbate (−Asc.) in a dose-dependent manner. Hydroxylation activity was determined using a multiwell VBC binding assay. (B) PHD-dependent hydroxylation reaction rate in the presence of 2mM ascorbate (control) or 2mM ascorbate combined with 5mM GSH (+5mM GSH). Shown are mean values ± SEM of triplicates. (C) Ascorbate determination by HPLC before and after 1 hour of PHD2-dependent hydroxylation reaction (left panels). Ascorbate content is only slightly decreased after 1 hour of incubation and independent of target hydroxylation (right panel). Shown are mean values ± SEM of 3 independent experiments normalized to values measured at time point zero.

increased the activity of all 3 untagged PHD isoforms, even in the absence of ascorbate (Figure 2A). Addition of GSH (+ 5mM in Figure 2B) to 2mM ascorbate increased the reaction rate compared with ascorbate alone (control in Figure 2B), suggesting 2 independent reaction modes of GSH, one mode replacing vitamin C and an additional mode enhancing the reaction rate. Of note, only minor changes in ascorbate oxidation were found before and after 1 hour of PHD2-mediated substrate hydroxylation (Figure 2C). The minor decrease in reduced ascorbate was probably because of air-dependent oxidation rather than enzymatic consumption because it was independent of the presence of the hydroxyl-acceptor substrate (Figure 2C right panel). In conclusion, as shown previously for C-P4H,[27] ascorbate is not consumed during coupled PHD-catalyzed hydroxylation reactions.

GSH decreases HIF activity in $CoCl_2$-treated hepatoma cells

In cell culture models, Co(II) and Ni(II) have been shown to substantially decrease cellular ascorbate content by catalyzing ascorbate oxidation to dehydroascorbate followed by irreversible hydrolysis to diketogulonate.[28] Interestingly, exogenous ascorbate administration completely blunted the Co(II)-induced hypoxic response in lung epithelial cells.[29] To test whether GSH could similarly compensate for reduced ascorbate levels after Co(II) stimulation, HIF transcriptional activity was further studied in HepG2 hepatoma cells stably transfected with an HIF-dependent luciferase reporter gene (HRG1 cells). We first determined the concentrations of $CoCl_2$ required to activate HIF-dependent reporter gene expression to a similar extent as exposure of the cells to 2% oxygen or the hypoxia mimicking iron chelator Dfx (Figure 3A). Subsequently, HRG1 cells were treated with 100μM $CoCl_2$ or 50μM Dfx under 21% or 2% oxygen. Control cells were kept at

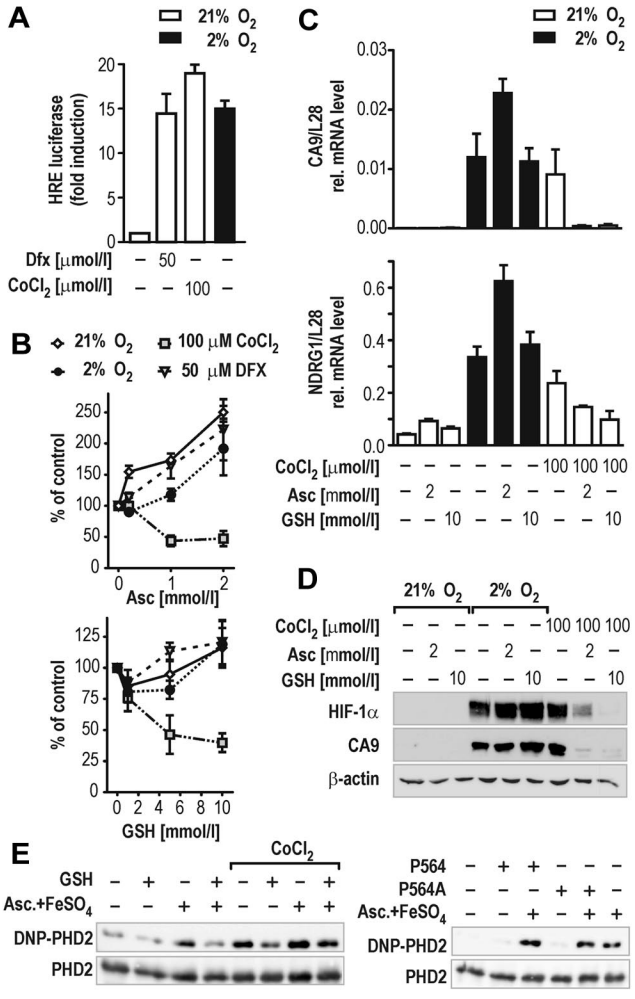

Figure 3. GSH impairs HIF activation in cells. (A) Induction of HIF-dependent luciferase reporter gene activity in stably transfected HRG1 hepatoma cells by 2% O_2, 50μM Dfx, or 100μM $CoCl_2$ for 24 hours. (B) Effects of ascorbate (Asc, 0.2-2mM; top panel) or GSH (1-10mM; bottom panel) in combination with hypoxia, Dfx, or $CoCl_2$ treatment on HIF-dependent luciferase activity relative to the protein concentration of the lysates. Shown are mean values ± SEM of 3 independent experiments normalized to the reporter activity in the absence of either ascorbate or GSH (control). (C) *CA9* and *NDRG1* HIF target gene mRNA levels in HRG1 cells after treatment with 2mM ascorbate or 10mM GSH combined with 2% O_2 or 100μM $CoCl_2$ for 24 hours. Shown are mean values ± SEM of 3 independent experiments relative to the mRNA content of ribosomal protein L28. (D) HIF-1α and CA9 protein levels in HRG1 cells after treatment with 2mM ascorbate or 10mM GSH combined with 2% O_2 or 100μM $CoCl_2$. (E) OxyBlot analyses of recombinant PHD2 protein carbonylation. GSH (5mM) reduced PHD2 carbonylation by either 2mM ascorbate/10μM $FeSO_4$ or 100μM $CoCl_2$ (left panel). PHD2 oxidation is independent of target hydroxylation as shown by using a wild-type or a Pro564Ala mutant HIF-1α hydroxyl-proline acceptor peptide in in vitro hydroxylation reactions (right panel).

ambient oxygen concentrations. All cells were cotreated with 1 to 10mM GSH or 0.2 to 2mM ascorbate. Indeed, ascorbate and GSH reduced HIF activity exclusively in $CoCl_2$-treated HRG1 cells (Figure 3B). A substantial increase of HIF activation was noted particularly when cells were treated with 2mM ascorbate, which might be explained by the pro-oxidative function ascorbate exerts if applied at high concentration (Figure 3B top panel).[30] In line with these observations, both ascorbate and GSH reduced the expression levels of the endogenous HIF target genes *CA9* and *NDRG1*[3] only in cells treated with $CoCl_2$, whereas 2mM ascorbate enhanced hypoxic activation of both genes by almost 2-fold (Figure 3C). To evaluate whether these effects reflected differential activities of cellular PHD enzymes, HIF-1α protein accumulation was analyzed by immunoblotting. As expected, only $CoCl_2$-stabilized HIF-1α was down-regulated by cotreatment with ascorbate or GSH (Figure 3D). Interestingly, 10mM GSH was a more potent inhibitor of $CoCl_2$-induced HIF-1α stabilization than 2mM ascorbate, a concentration that showed saturated inhibition of the HIF-reporter in the same cell line (Figure 3B top panel).

GSH protects PHD2 from metal-catalyzed oxidation

Enzymatic activity of the PHDs is sensitive to reactive oxygen species and transition metal ions.[31] However, the mechanism(s) by which reactive oxygen species or metal ions inhibit hydroxylase activity remained speculative. Besides its general antioxidative properties as radical scavenger, vitamin C actively interferes with the oxidation state of metal ions by serving as electron donor in a redox reaction. As such, it largely differs from GSH that is a major cellular antioxidant protecting cysteinyl and methionyl residues in proteins from oxidative modifications. Both $CoCl_2$ and H_2O_2 inhibited all 3 PHD isoforms in vitro, with PHD2 being slightly more resistant to $CoCl_2$ (supplemental Figure 3). To directly determine protein oxidation by these compounds, carbonyl group formation in PHD2 was estimated by OxyBlot technology. As shown in Figure 3E (left panel), 2mM ascorbate and 10μM $FeSO_4$ (as used in the standard reaction buffer for in vitro hydroxylation) substantially increased carbonylation of recombinant PHD2 during 1 hour of hydroxylation reaction. Surprisingly, $CoCl_2$ increased carbonylation of PHD2 whether ascorbate/$FeSO_4$ was present or not. After addition of 5mM GSH, oxidation of PHD2 by ascorbate/$FeSO_4$, $CoCl_2$, and H_2O_2 was markedly reduced. To examine whether PHD2 protein oxidation is coupled to its dioxygenase activity, the reaction was performed in the presence of a mutant Pro564Ala HIF-1α peptide substrate. As shown in Figure 3E (right panel), PHD2 protein oxidation was independent of the presence of a hydroxylation acceptor proline, providing evidence that protein oxidation is not caused by the hydroxylation reaction cycle.

Cysteine 201 affects PHD2 hydroxylation activity

Recently, cysteine residue 201 within the catalytic domain of PHD2 has been identified as a surface-accessible, highly nucleophilic residue predominantly interacting with thiol compounds.[32] Moreover, Cys201 and Cys208 were proposed to provide an additional metal binding site in PHD2.[33] Both, Cys201 and Cys208 are highly conserved among all 3 human and mouse PHD isoforms (Figure 4A). To investigate the functional relevance of Cys201, recombinant PHD2, wild type, or Cys201Ser mutant was purified from Sf9 cells. Surprisingly, the Cys201Ser mutation significantly ($P < .0001$) increased the PHD2 reaction rate by 2.5-fold (Figure 4B top panel). Equal concentrations of wild-type and mutant PHD2 proteins were confirmed by Coomassie staining of the undiluted stock solutions and immunoblotting of the diluted assay solutions (Figure 4B bottom panel). To further test the hypothesis that the Cys201Ser mutation might protect the PHD2 enzyme from oxidative damage, the effect of H_2O_2 on hydroxylation activity was measured. As shown in Figure 4C, the half-maximal inhibitory concentration for H_2O_2 was roughly 5-fold higher for the Cys201Ser mutant compared with wild-type PHD2 (half-maximal inhibitory concentration of 8.9×10^{-6} and 4.3×10^{-5} M H_2O_2 for wild-type and Cys201Ser mutant PHD2, respectively). We further tested whether the H_2O_2-mediated loss of PHD2 activity could be rescued by sequential addition of GSH. For both enzyme preparations,

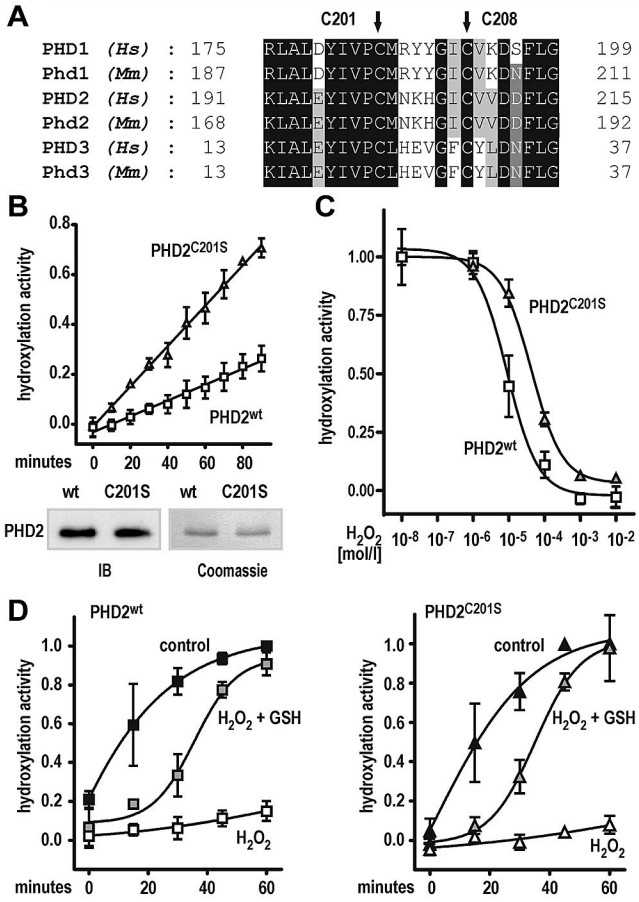

Figure 4. A Cys201Ser mutation enhances PHD2-dependent hydroxylation reaction rate and protects from protein oxidation. (A) Conservation of cysteine residues (Cys201 and Cys208 in human PHD2) in all 3 human (*Hs*) and mouse (*Mm*) PHD isoforms. (B) Increased reaction rate of Cys201Ser mutant PHD2 as measured by the hydroxylation-dependent VBC binding assay. Shown are mean values ± SEM of 3 independent experiments. Linear regression analyses were performed, revealing highly different slopes ($P < .0001$). (C) The Cys201Ser mutation confers resistance of PHD2 to H_2O_2-mediated inhibition of hydroxylation activity. Shown are mean values ± SEM of a representative experiment performed in triplicates. (D) GSH can rescue PHD2 wild type and Cys201Ser hydroxylation activities after H_2O_2-mediated enzyme damage. In brief, enzyme preparations were preincubated with 1mM H_2O_2 for 30 minutes (H_2O_2) or left untreated for a similar period (control). For rescue experiments, enzymes after H_2O_2 treatment were incubated with 5mM GSH (H_2O_2 + GSH) for 15 minutes. Hydroxylation reactions were carried out at standard assay conditions for 60 minutes. Note that all reactions contained 2mM ascorbate freshly added when hydroxylation reactions were started. Data are given as mean values ± SEM of 3 independent experiments normalized to hydroxylation activities of control reactions obtained after 60 minutes.

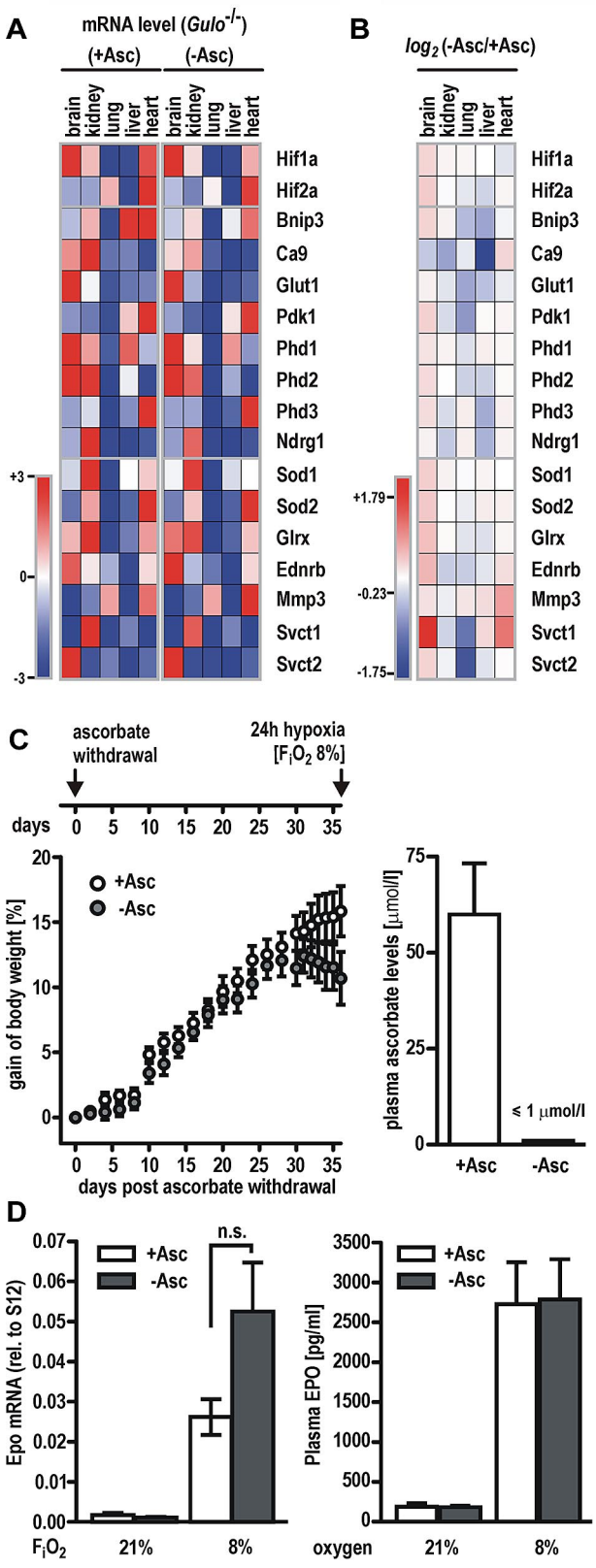

Figure 5. Hypoxic response is fully functional in vitamin C–depleted *Gulo*$^{-/-}$ mice. *Gulo*$^{-/-}$ male mice received a diet with (+Asc) or without (−Asc) ascorbate for 5 weeks. (A) Transcript levels of HIF target genes (*Bnip3*, *Ca9*, *Glut1*, *Pdk1*, *Phd2*, *Phd2*, *Phd3*, and *Ndrg1*) as well as genes involved in antioxidative defense (*Sod1*, *Sod2*, and *Glrx*), the ascorbate transporters *Svct1* and *Svct2*, or oxygen-independent genes (*Ednrb*, *Mmp3*, and *Phd1*) were quantified by RT-qPCR in brain, kidney, lung, liver, and heart. Values are expressed relative to S12 mRNA levels and visualized in a heatmap (Genepattern; Broad Institute). Lowest and highest mRNA levels of each gene were arbitrarily defined as −3 (dark blue) and +3 (dark red), respectively. (B) Heatmap of gene expression changes following a vitamin C–deficient diet. Log_2 (−Asc/+Asc) ratios revealed that the majority of HIF target genes remained either unchanged or showed a slightly reduced expression pattern. Data ranged from −1.75 (dark blue) and +1.79 (dark red), respectively. (C) Scheme depicting the experimental setup for hypoxic experiments with vitamin C–depleted *Gulo*$^{-/-}$ animals (top panel). Relative gain of body weight of *Gulo*$^{-/-}$ mice (n = 11 animals per group) after ascorbate withdrawal (−Asc) or ascorbate supplementation (+Asc) for 36 days (left panel). Ascorbate levels in the plasma of *Gulo*$^{-/-}$ mice (n = 6 animals per group) after 5 weeks of ascorbate withdrawal compared with mice kept on an ascorbate-supplemented diet (right panel). (D) Epo mRNA (left panel) and circulating EPO protein (right panel) levels in *Gulo*$^{-/-}$ mice maintained on a diet with (+Asc) or without (−Asc) for 5 weeks followed by exposure to 8% or 21% oxygen for 24 hours. Data represent mean values ± SEM derived from at least 5 animals per group; n.s., indicates not significant.

preincubation with 1mM H_2O_2 for 30 minutes inhibited subsequent substrate hydroxylation reactions, despite 2mM ascorbate being freshly added to start the hydroxylation reaction (Figure 4D). Interestingly, further addition of 5mM GSH could similarly reactivate both enzyme preparations, although reaction kinetics was substantially slower for the reactivated enzymes (Figure 4D).

Oxygen sensing is fully functional in ascorbate-deficient *Gulo*$^{-/-}$ mice

Gulo$^{-/-}$ mice received a diet with or without vitamin C for 5 weeks. Transcript levels of known HIF target genes (Bnip3, Ca9, Glut1, Pdk1, Phd2, Phd3, and Ndrg1) as well as genes involved in antioxidative defense (Sod1, Sod2, and Glrx), the ascorbate transporters Svct1 and Svct2, or oxygen-independent genes (Ednrb, Mmp3, and Phd1) were determined by RT-qPCR in brain, lung, kidney, heart, and liver. Similar tissue-specific expression levels for most genes involved in different pathways were observed in both groups (Figure 5A). Conclusive with our finding from cell culture experiments, expression levels of most of the HIF target genes remained largely unaffected by vitamin C deficiency or even showed reduced levels (blank and blue squares, respectively, in the heatmap shown in Figure 5B). Expression of the ascorbate transporter Svct1, however, was moderately induced in animals fed without ascorbate, possibly reflecting compensatory mechanisms for ascorbate deprivation.

To further test whether the absence of ascorbate limits PHD function under hypoxic conditions, *Gulo*$^{-/-}$ males were deprived of vitamin C for 5 weeks, whereas control animals were supplemented with vitamin C. As described previously,[18] the animals developed a scorbutic phenotype marked by a substantial loss of body weight after 35 days on a ascorbate-free diet, indicating that systemic stores of vitamin C have been exhausted (Figure 5C left panel). In line with this observation, plasma ascorbate levels in *Gulo*$^{-/-}$ mice fed an ascorbate-free diet were below the detection limit (≤ 1µM), whereas plasma of control animals contained 40.3 to 123.8µM vitamin C (Figure 5C right panel), corresponding to ascorbate plasma levels in healthy humans.[34]

After 5 weeks of vitamin C deprivation, mice were breathing 8% oxygen for an additional period of 24 hours, whereas control groups were kept under ambient oxygen concentration (see scheme in Figure 5C). Both ascorbate-deficient and supplemented animals responded to the hypoxic treatment with a robust induction of Epo mRNA in the kidney that was higher in *Gulo*$^{-/-}$ males fed without ascorbate, although differences did not reach the level of significance ($P = .07$, Student t test; Figure 5D left panel). Circulating EPO protein levels in mouse plasma were induced by hypoxic exposure to a similar extent in both groups (Figure 5C right panel).

Of note, no significant changes of the red cell lineage hematologic parameters were observed in *Gulo*$^{-/-}$ mice after 5 weeks of ascorbate depletion, indicating that the oxygen transport capacity was similar in both treatment groups. Hypoxic increases in hematocrit values are known to be delayed and reach the level of significance not before 72 hours of continuous exposure to hypoxia,[35] explaining the lack of an increase of either hematocrit values or red blood cell counts in our experimental setting with a hypoxic period of only 24 hours (supplemental Table 2).

Discussion

Ascorbic acid and ferrous iron have been reported as essential cofactors for PHD-dependent HIFα hydroxylation in vitro.[13] Unexpectedly, we found a fully functional cellular oxygen-sensing pathway in HeLa cells maintained under strictly ascorbate-free culture conditions, indicating that ascorbate is dispensable for HIFα hydroxylation in vivo. In a search for the nature of antioxidative compounds substituting for ascorbate during prolyl-4-hydroxylation, we identified GSH as a potent activator of all 3 PHDs in vitro, increasing HIFα peptide hydroxylation in a dose-dependent manner. Notably, ascorbate and GSH are the most abundant reducing compounds within eukaryotic cells.[36] Given the distinct antioxidative properties of vitamin C and GSH, the 2 compounds might affect prolyl-4-hydroxylation by different ways. Ascorbate might be required to reduce occasionally oxidized ferric Fe(III) generated in the active center of PHDs by uncoupled reaction cycles as it has been described for C-P4H.[27] However, to the best of our knowledge, no experimental evidence has been reported for enzymatic activity of PHDs in the absence of a hydroxyl-acceptor substrate. Strikingly, the major iron form bound to purified PHD2 is ferrous Fe(II) even when purified under oxygenated conditions,[37] arguing against an essential role of ascorbate in reducing PHD iron. In support of this notion, iron and 2-oxoglutarate have been reported to copurify with 50% and 5%-10% of PHD2, respectively, whereas ascorbate did not copurify at all.[37] Recent work by Flashman et al[38] showed that ascorbate does not directly interact with the catalytic domain of PHD2; however, its intrinsic ene-diol–reducing moiety was found to be important to promote hydroxylation by PHD2.

GSH fully stimulated in vitro PHD hydroxylation activity only at rather high concentrations, which is in line with findings reported previously for N-terminally truncated PHD2.[38] The millimolar GSH concentrations used in our study reflect physiologically relevant levels of this compound in living cells.[39] Moreover, we found that ascorbic acid is not consumed by coupled substrate hydroxylation, suggesting that exogenously added GSH does not simply regenerate potentially copurified oxidized dehydroascorbate. Our data rather favor an alternative function of GSH by preventing oxidative damage to the enzyme itself. Physiologic concentrations of GSH were able to reduce transition metal- or peroxide-induced PHD enzyme carbonylation. Despite being generally referred to as antioxidant, ascorbate, together with oxygen and transition metals such as Fe(III) or Cu(II), also exerts pro-oxidative effects by generating hydroxyl radicals in a Fenton-like reaction.[40] Indeed, we found increased PHD2 carbonylation by ascorbate-iron in vitro, suggesting that GSH might protect PHDs from the adverse effects of ascorbate. In line with 2 distinct reaction modes, addition of GSH to hydroxylation reactions containing saturating ascorbate concentrations markedly increased the hydroxylation rate of PHDs in vitro. Of note, Co(II) induced ascorbate depletion, as suggested for cultured cells,[28,29] cannot account for PHD inhibition in our cell-free assays, because we showed previously that Co(II) only inefficiently catalyses ascorbate oxidation by air under these assay conditions.[16] Direct interference of Co(II) with the enzymes is supported by the observation that Co(II) strongly carbonylated purified PHD2 even in the absence of ascorbate. Moreover, ascorbate and GSH exclusively blunted Co(II)-induced HIF activation in our cellular models, demonstrating a complementary function of GSH and ascorbate in oxygen sensing by living cells. Although simple chelation of Co(II) by ascorbate and GSH cannot be fully excluded in cell culture experiments, it should be mentioned that metal chelators naturally occurring in serum (eg, histidine, glutamic acid, and albumin but also GSH) are essential to facilitate Co(II)-induced ascorbate oxidation, because "free" Co(II) is unable to directly oxidize ascorbate in simple aqueous solutions at neutral pH (for a review, see Salnikow and Kasprzak[28]). Thus, the actual redox potential of the ion in such ternary complexes —rather than the intracellular concentration of "free" metal—determines its efficacy to act as a "hypoxia mimetic."

One of the specific GSH functions is to prevent or reduce inappropriate disulfide bond formation. Two recent reports identified PHD2 cysteinyl residues Cys201 and Cys208 to be highly nucleophilic and surface accessible.[32,33] Moreover, crystallographic analyses predicted that these 2 cysteines might form disulfide bonds.[32,33] Cys201Ser mutation protects recombinant PHD2 from oxidative damage in vitro and results in a 2.5-fold higher specific hydroxylation activity. One might speculate that a certain fraction of wild-type PHD2 enzyme constantly undergoes oxidative modification of Cys201, leading to reduced activity. As such, PHD enzymes could combine oxygen- and redox-sensing properties, providing a possible explanation for previous work on redox factors modulating PHD activity.[41-43] However, potentiation of PHD activity by GSH clearly involves mechanisms distinct from Cys201 oxidation, because both wild-type and Cys201Ser mutated PHD2 enzymes could be efficiently reactivated from H_2O_2-induced damage by GSH.

Translating our biochemical and cellular findings into a systemic context, we did not observe marked alterations in HIF target gene expression after dietary vitamin C deprivation in *Gulo*$^{-/-}$ mice, providing evidence that other antioxidants might substitute for vitamin C in vivo. Accordingly, the hypoxic response of *Gulo*$^{-/-}$ mice with low or undetectable ascorbate in the plasma was similar to that of mice receiving an ascorbate-supplemented diet. Consistent with antioxidant redundancy in vivo, a study using the same animals backcrossed to a BALB/c genetic background found vitamin C–independent de novo synthesis of collagen in allografted tumors and unchanged levels of hydroxyproline-collagen. Dermal hydroxyproline content of collagen was even increased.[44] Of note, treatment of vitamin C–deprived guinea pigs with a cell-permeable GSH monoethyl ester significantly attenuated the severity of scurvy-related symptoms, also suggesting a cooperative function of both antioxidants for P4H function in vivo.[45] Moreover, *Gulo*$^{-/-}$ mice have increased levels of total glutathione in brain and liver, possibly explained by a compensatory mechanism for antioxidative defense in these animals.[46]

Interestingly, tumor growth and angiogenesis were retarded in a syngenic tumor model using vitamin C–deprived *Gulo*$^{-/-}$ mice, but no changes were observed for HIF-1α protein levels in the respective tumor tissue.[47] The possible value of vitamin C in cancer therapy recently experienced a renaissance, because it has been shown that pharmacologic doses of vitamin C decrease growth of

tumor xenografts in mice by increasing peroxide levels in neoplastic tissue.[30,48] Such tumoricidal effects of antioxidants might, at least partially, involve destabilization of HIFα after increased hydroxylase activity.[49,50] Our data support a model of cooperative function of GSH and vitamin C in regulating the efficiency of PHD oxygen sensors. Translated to chemotherapy of cancers, combined treatment with both clinically approved molecules might even boost their antitumorigenic function.

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Authorship

Contribution: K.J.N. designed and performed experiments, analyzed data, and wrote the manuscript; K.J.N., N.M., P. Schläfli, and P. Spielmann performed in vivo experiments; R.H.W. designed experiments and wrote the manuscript; and D.P.S. designed experiments, analyzed data, and wrote the manuscript.

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Table S1. **Sequence of human and mouse primers used for RT-qPCR measurements.**

Ca9, carbonic anhydrase IX; Ndrg1, N-myc downstream regulated gene 1; L28, ribosomal protein L28; Hif1a, hypoxia inducible factor 1, α subunit; Hif2a, hypoxia inducible factor 2, α subunit; Bnip3, BCL2/adenovirus E1B interacting protein 1; Glut1, glucose transporter, member 1; Pdk1, pyruvate dehydrogenase kinase, isoenzyme 1; Phd1-3, prolyl-4-hydroxylase domain 1-3; Sod1, superoxide dismutase 1; Sod2, superoxide dismutase 2; Glrx, glutaredoxin; Ednrb, endothelin receptor type B; Mmp3, matrix metallopeptidase 3; Svct1, sodium-dependent vitamin C transporter 1; Svct2, sodium-dependent vitamin C transporter 2; Epo, erythropoietin; S12, Ribosomal protein S12.

	Species	Accession No.	Forward primer	Reverse primer
CA9	human	NM_001216	5′- gggtgtcatctggactgtgtt -3′	5′- cttctgtgctgccttctcatc -3′
NDRG1	human	NM_006096	5′- atgtacccctccatggatca -3′	5′- tgtggaccacttccacgtta -3′
L28	human	NM_000991	5′- gcaattccttccgctacaac -3′	5′- tgttcttgcggatcatgtgt -3′
Hif1a	mouse	NM_010431	5′- ggttccagcagacccagtta -3′	5′- aggctccttggatgagcttt -3′
Hif2a	mouse	NM_010137	5′- taaagcggcagctggagtat -3′	5′- actgggaggcatagcactgt -3′
Bnip3	mouse	NM_009760	5′- gctcccagacaccacaagat -3′	5′- tgagagtagctgtgcgcttc -3′
Ca9	mouse	NM_139305	5′- gctgtcccatttggaagaaa -3′	5′- ggaaggaagcctcaatcgtt -3′
Glut1	mouse	NM_011400	5′- tctctgtcggcctctttgtt -3′	5′- gcagaagggcaacaggatac -3′
Pdk1	mouse	NM_172665	5′- ggcggctttgtgatttgtat -3′	5′- acctgaatcgggggataaac -3′
Phd1	mouse	NM_053208	5′- ttgcctgggtagaaggtcac -3′	5′- gctcgatgttggctaccact -3′
Phd2	mouse	NM_053207	5′- agccatggttgcttgttacc -3′	5′- ctcgctcatctgcatcaaaa -3′
Phd3	mouse	NM_028133	5′- caacttcctcctgtccctca -3′	5′- ggctggacttcatgtggatt -3′
Ndrg1	mouse	NM_010884	5′- tcaagatggcagactgtgga -3′	5′- gttgggggtgatgttgagac -3′
Sod1	mouse	NM_011434	5′- ccagtgcaggacctcatttt -3′	5′- cacctttgcccaagtcatct -3′
Sod2	mouse	NM_013671	5′- ggccaagggagatgttacaa -3′	5′- gaaccttggactcccaca -3′
Glrx	mouse	NM_053108	5′- aacaacaccagtgcgattca -3′	5′- atctgcttcagccgagtcat -3′
Ednrb	mouse	NM_007904	5′- cagtcttctgcctggtcctc -3′	5′- ggactgcttttcctcaaacg -3′
Mmp3	mouse	NM_010809	5′- ctatacgagggcacgaggag -3′	5′- ccacccttgagtcaacacct -3′
Svct1	mouse	NM_011397	5′- tctttggcctcacactaccc -3′	5′- tccttttttaccatgcccatc -3′
Svct2	mouse	NM_018824	5′- tgccaggaagggtgtacttc -3′	5′- ccggtaccaaatatgccatc -3′
Epo	mouse	NM_007942	5′- ggccatagaagtttggcaag -3′	5′- cctctcccgtgtacagcttc -3′
S12	mouse	NM_011295	5'- gaagctgccaaagccttaga -3'	5'- aactgcaaccaaccaccttc -3'

Table S2. Blood parameters of *Gulo*$^{-/-}$ mice. All mice received ascorbate free provender for 5 weeks. Control animals received vitamin C via their drinking water. Heparinized whole blood samples were collected from mice kept at ambient oxygen tension or in a hypoxic environment (8% oxygen for 24 hours) by cardiac puncture. The values represent mean values ± SD of at least three animals per group as indicated.

	20% oxygen [Fi O_2]		**8% oxygen [Fi O_2]**	
	+Asc (n=3)	**-Asc (n=4)**	**+Asc (n=4)**	**-Asc (n=5)**
Hemoglobin(g/dl)	12.57±0.6807	13.43±0.3304	12.33±2.022	12.20±1.030
Hematocrit (%)	40.23±1.528	42.88±0.3862	40.68±6.247	39.72±3.492
Erythrocytes (10^6/µl)	8.777±0.5724	9.250±0.09019	8.625±1.275	8.496±0.6687
Retikulocytes (10^3/µl)	260.3±42.34	227.0±21.46	295.3±106.4	273.2±53.18

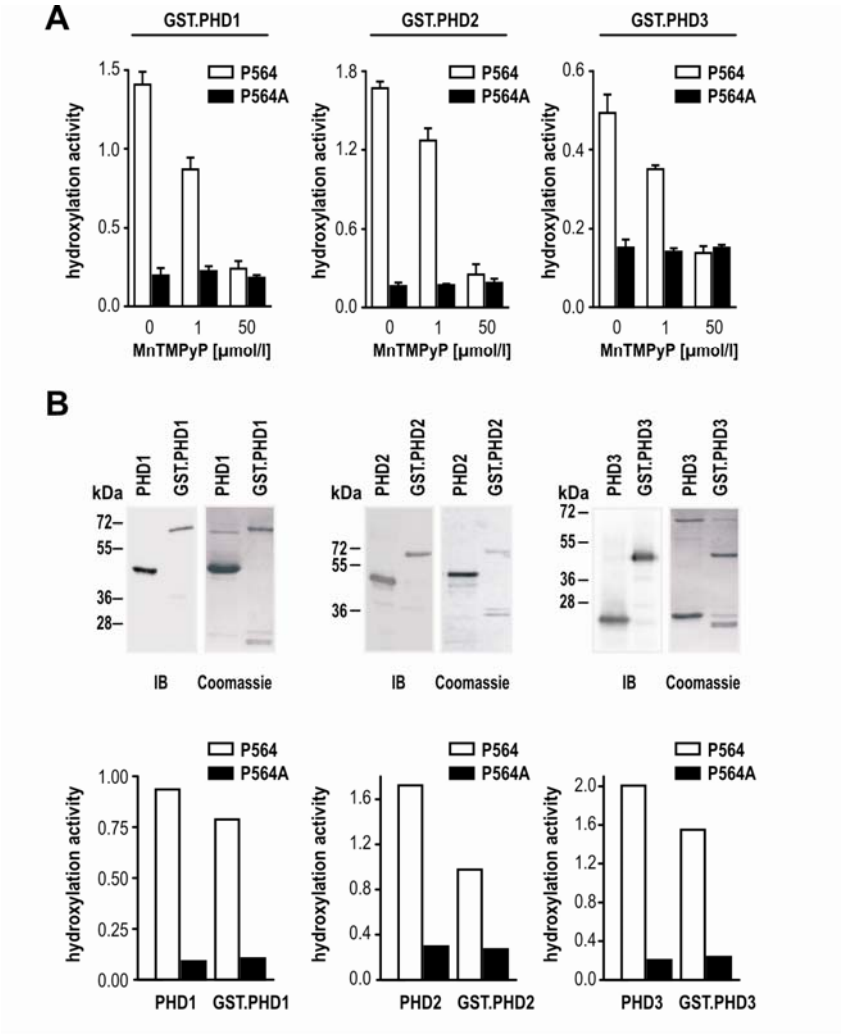

Figure S1. Inhibition of PHDs by MnTMPyP and *in vitro* hydroxylation activity of untagged PHD1-3. (A) Inhibition of PHD1-3 hydroxylation activity by Mn(III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP) measured by an *in vitro* hydroxylation assay. The presence of 50 µM MnTMPyP completely abolished hydroxylase activity of all three PHDs. Shown are mean values ± SEM of an experiment performed in triplicates. (B) Comparison of the purity (upper panels. IB, immunoblotting; Coomassie, protein staining by Coomassie blue) and hydroxylation activity (lower panels) of GST-tagged and untagged PHD1-3 enzyme preparations. Representative experiments performed in duplicates are shown. A Gateway-Technology compatible expression vector for GST fusion proteins bearing the PreScission protease cleavage site was generated by introducing a Leu-Glu-Val-Leu-Phe-Gln-Gly-Pro peptide into pDEST20 (Invitrogen) by site-directed mutagenesis. The C201S mutation

was introduced into wild-type PHD2 by site-directed mutagenesis. PHD1, PHD2 and PHD3 expression vectors were generated by homologous recombination with respective Entry vectors (Invitrogen). For expression of recombinant proteins, Sf9 cells were infected with baculovirus stock and cultured in Grace's insect medium at 27ºC in a humidified incubator for 96-110 hours. Cells were collected by centrifugation and lysed in ice-cold 0.1% NP-40, 10 mM Tris-HCl pH 7.5, 100 mM NaCl, 100 mM glycine and 10 mM DTT. Cleared lysates were incubated with PBS-equilibrated GSH-sepharose beads (GE Healthcare) for 2 hours at 4°C with gentle agitation. For cleavage, beads were washed twice with PBS and equilibrated twice with PreScission cleavage buffer (50 mM Tris-Cl pH 7.0, 150 mM NaCl). PreScission protease (GE Healthcare) was added to the protein-bound glutathione-sepharose beads (80 U in 960 ml cleavage buffer per 1 ml of bead volume) and incubated for 5 hours at 4ºC.

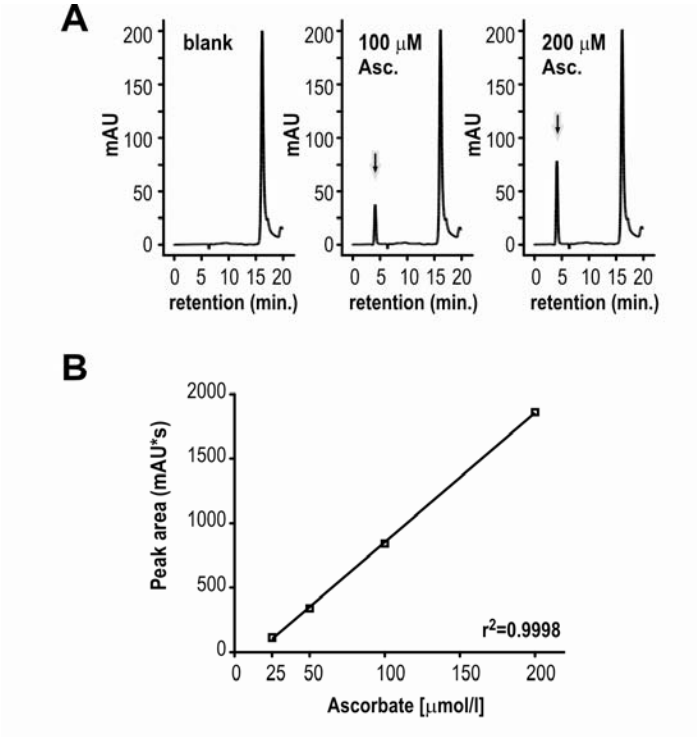

Figure S2. HPLC determination of ascorbate concentrations. (A) Exemplary chromatograms derived from samples of blank (60 mM phosphoric acid, left), or spiked standard solutions containing 100 µM (middle) and 200 µM (right) ascorbate, respectively. Probes were loaded on a Nucleosil C18 column and eluted applying an acetonitrile gradient (0-60%). Arrows indicate the position of the peaks specific for ascorbate. (B) Standard curve as obtained after plotting the peak areas of ascorbate elutions (given in arbitrary units; AU) against known ascorbate concentrations in spiked samples. Regression analysis was performed using Prism 4.0 GraphPad software.

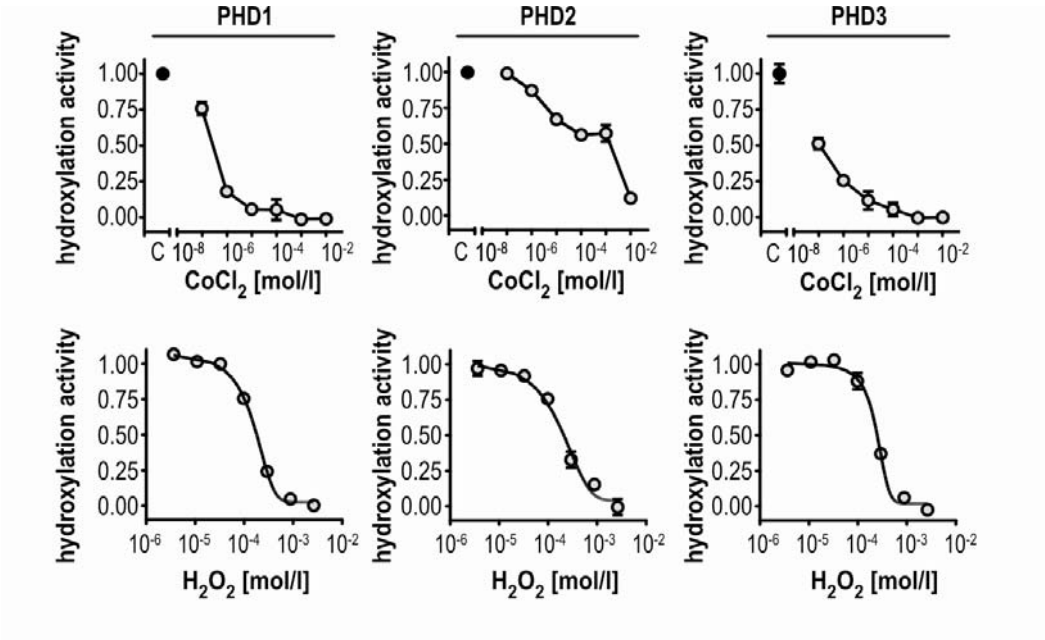

Figure S3. $CoCl_2$ and H_2O_2 inhibit the *in vitro* hydroxylation activity of PHD1-3.

Dose-dependent inhibition of PHD activity by $CoCl_2$ (upper panel) and H_2O_2 (lower panel). Shown are mean values ± SEM of a representative experiment performed in triplicates.